REMARKS/ARGUMENTS

With this amendment, claims 1-8, 16, and 28-29 are pending. Claims 9-15 and 17-27 are cancelled. For convenience, the Examiner's rejections are addressed in the order presented in the June 14, 2005,Office Action.

I. Rejections under 35 U.S.C. §112, first paragraph, written description

Claims 1-8, 16, 28, and 29 are rejected for allegedly containing subject matter that was not described in the specification at the time of filing. According to the Office Action limitations such as "the antibody inhibits growth of the malignant cell that expresses the frizzled 5 receptor" in claims 1 and 16 and "wherein the antibody is effective for immunotherapy of a malignant cell that overexpresses the frizzled 5 receptor" in claim 28 are new matter. Office Action at page 2. The Office Action further states that the specification supports only a genus of antibodies against any member of frizzled products, but not an antibody directed to a particular frizzled protein. Office Action at page 5. Applicants respectfully traverse the rejection.

According to the MPEP, there is no *in haec verba* requirement for newly added claim limitations and those limitations can be supported by express, implicit, or inherent disclosure. MPEP 2163 IB. As discussed below, the specification as filed provides ample support for the for the claimed antibodies.

Original claim 1 recites a "purified antibody for modulating a biological activity of a malignant cell that expresses a frizzled receptor, wherein said antibody specifically binds to at least one epitope in an extracellular domain of the frizzled receptor expressed on the malignant cell." The claim limitation "modulating a biological activity of a malignant cell" is defined in the specification at page 22, lines 3-6, and, refers to the "ability of the antibody to effect cellular function." These effects include cell growth inhibition, the ability to elicit a cytotoxic response to the malignant cell, or other negative effects on the malignancy. Applicants assert that the cell growth inhibition recited in the specification is essentially equivalent to the now claimed ability of the antibody to inhibit cell growth.

Original claim 10 depends from original claim 1, and thus includes all the limitations of claim 1, including, *e.g.*, "modulating a biological activity of a malignant cell". As discussed above, this phrase includes inhibition of growth of a malignant cell. Original claim 10 further recites that the frizzled receptor extracellular domain is 80% homologous to an amino acid selected from a group of SEQ ID NOs: 61, 62, 63, 64, 66, **68**, 69, 71, 73, 75, and 77. Table 2 at page 11 discloses the SEQ ID NO used to denote an extracellular domain for any specific frizzled protein disclosed in the specification and the specific sequences of the extracellular domains are provided in Figure 8. SEQ ID NO:**68** is the extracellular domain for frizzled 5. Thus, original claims 1 and 10 and the specification provide support for specific antibodies against an extracellular domain of frizzled 5 that inhibit growth of a malignant cell.

Moreover, the specification goes on to discloses that 10 human frizzled proteins are known and, on page 9 at Table 1, lists the gene encoding each specific human frizzled proteins. Sequences of specific frizzled proteins are provided in Figure 8, which also discloses the specific extracellular domains of each frizzled protein. The specification also includes Table 2, which lists the SEQ ID NOs for each full length frizzled proteins 1-10, and each fzd protein extracellular domain, including the amino terminal domain and loops 1, 2, and 3. Clearly the applicants took the time to include disclosure of each individual human frizzled protein and its extracellular domain in order to support claims to each individual species, as well as the genus. Thus, the term frizzled protein is used within the specification to encompasses both the genus of frizzled proteins and the individual members of the genus disclosed in the specification. No Office Action, including the most recent, has provided a credible argument that disclosure of individual protein sequences and domain sequences within those proteins, does not, in fact, support claims to the individual protein domains and thus, to the antibodies that bind to them. Thus, Applicants reiterate that the specification as filed provides more than adequate support for the claimed frizzled 5 antibodies.

In view of the above arguments, withdrawal of the rejection for alleged lack of written description is respectfully requested.

III. Rejections under 35 U.S.C. §112, first paragraph, enablement

Claims 1-8, 16, 28, and 29 are rejected under 35 U.S.C. §112, first paragraph, as allegedly lacking enablement. According to the Office Action, the specification does not enable one of skill to make and/or use the invention commensurate in scope with the claims. The Office Action also alleges that undue experimentation is required to practice the claimed invention. Applicants respectfully traverse the rejection.

Factors such as the amount of guidance presented in the specification and the presence of working examples must be considered to determine whether undue experimentation is required to practice the claimed invention. *See, e.g., Ex Parte Forman*, 230 USPQ 546 (Bd. Pat. App. & Int. 1985) and *In re Wands*, 8 USPQ2d 1400 (Fed. Cir. 1988). As described in *Wands*, "a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed." *Wands*, USPQ2d at 1404, quoting *In re Jackson*, 217 USPQ 804 (Bd. Pat. App. & Int. 1982). Moreover, "[a] patent need not teach, and preferably omits, what is well known in the art." MPEP 2164.01 *citing In re Buchner*, 18 USPQ2d 1331, 1332 (Fed. Cir. 1991); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 231 USPQ 81, 94 (Fed. Cir. 1986), *cert. denied*, 480 U.S. 947 (1987); *Lindemann Maschinenfabrik GMBH v. American Hoist & Derrick Co.*, 221 USPQ 481, 489 (Fed. Cir. 1984).

As set forth in the Manual of Patent Examining Procedure (MPEP) § 2164.01, "the test of enablement is not whether any experimentation is necessary, but whether... it is undue." Further, the "fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation" (citations omitted). Finally, claims reading on inoperative embodiments are enabled if the skilled artisan understands how to avoid inoperative embodiments. *See, e.g., In re Cook and Merigold*, 169 USPQ 299, 301 (C.C.P.A. 1971). Applicants respectfully assert that *In re Cook* is improperly construed to provide a minimum number of operative embodiments required for enablement.

The Office Action alleges that the specification does not provide a single species of the claimed invention, and further alleges that the disclosure of the exemplified Fzd-2 antibodies has no bearing on the operation of the claimed Fzd-5 antibodies. The Office Action

also expressed doubt that the claimed invention could ever be shown to exist. Office Action at page 9. The Office Action admits that the specification does teach that fzd proteins generally are involved in wnt/fzd signaling, but alleges that, in spite of disclosure of the amino acid sequences of the extracellular domain of each fzd protein, the specification teaches only how to make antibodies against the exemplified fzd-2 protein. Applicants respectfully assert that, based on the specification and the knowledge in the art at the time of filing, those of skill would be able to make and use the claimed antibodies directed against the extracellular domain of the fzd-5 protein.

Applicants were the first to provide teaching that antibodies against a specific fzd protein extracellular domain can be used to block that wnt fzd signaling pathway and thus, to inhibit growth or survival of cells that express the specific fzd protein. Applicants further taught in the specification that such antibodies against specific fzd extracellular domains can be used to inhibit growth or survival of malignant cancer cells. As discussed in the response filed on March 18, 2005, those of skill were aware of methods to produce antibodies against a specific protein sequence. Applicants provide methods to determine whether antibodies against specific fzd extracellular inhibit growth of cancer cells *in vitro*, at *e.g.*, Examples 3 and 4, and in a syngeneic mouse model at page 33, line 19 through page 34, line 5.

In support of the existence of Fzd-5 antibodies that inhibit growth or survival of a malignant cell, Applicants submit Exhibit A, Sen *et al.*, *Arthr. & Rheum.* 44:772-781 (2001) and Exhibit B, Weeraratna *et al.*, *Cancer Cell* 1:279-288 (2002). Applicants respectfully point out that Sen *et al.* was submitted to the USPTO in an IDS dated Aug. 29, 2002 and has already been considered by the Examiner. Sen *et al.* discloses synthesis of an antibody directed against the extracellular domain of the Fzd-5 protein. Sen *et al.* at page 773. The reference also discloses that this Fzd-5 antibody can be used to block the wnt/fzd signaling pathway in fibroblast-like synoviocyte cell lines, which model rheumatoid arthritis. Sen *et al.*, pages 777-779, Figures 4 and 5. The authors of Sen *et al.* (some also named inventors herein) provided the fzd-5 extracellular domain antibody to other researchers who, following the teaching of the application, demonstrated that the fzd-5 extracellular domain antibody can inhibit invasiveness of melanoma cells that express the fzd-5 protein, preventing metastasis of the malignant cells.

Weeraratna *et al.*, pages 282-283, Figure 5, and page 287. Thus, the specification as filed provides ample teachings on how to make and use the claimed fzd-5 antibodies.

The Office Action at pages 13-14 also alleges that another reference from some of the inventors, Rhee *et al.*, somehow demonstrates that the fzd-5 protein "might not be expressed on a malignant cell." The Office Action also alleges that Rhee *et al.* demonstrates that undue experimentation is required to determine whether a protein is a cancer antigen. This interpretation is not correct. Rhee *et al.* provide evidence of exactly the types of experimentation that is done in the field of immunotherapeutic, particularly cancer immunotherapeutics. Rhee *et al.* demonstrate that tumor cells are routinely assayed for expression of a potential antigen targeted by an immunotherapeutic. After confirmation that the appropriate protein is expressed, the therapeutic antibody is administered. Nothing in Rhee *et al.* suggests that this type of analysis is anything more than routine experimentation.

Based on the knowledge of antibody techniques in the art or their disclosure in the specification, those of skill would understand how to avoid inoperative embodiments. Applicants respectfully remind the Examiner that routine screening for operative embodiments is not precluded by the statute. For example, in *In re Wands*, the Federal Circuit agreed that screening is appropriate in technologies that do so routinely to identify embodiments with desired characteristics. *See, e.g., In re Wands*, 8 USPQ2d 1400, 1406-7 (Fed. Cir. 1988). Here, those of skill routinely assay tumors for expression of signaling proteins, such as fzd proteins.

In order to establish a prima facie case of lack of enablement, the Examiner has the burden to establish a reasonable basis to question the enablement provided for the claimed invention. *In re Wright*, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993). The Examiner has not provided any credible reason why those of skill would not be able to make and use antibodies against the fzd-5 protein to inhibit growth of cancer cells, based on the disclosure of the specification and the knowledge of the art at the time of filing. Applicants respectfully assert that based on the disclosure of the specification and the state of the art at the time of filing, the claimed antibodies are enabled.

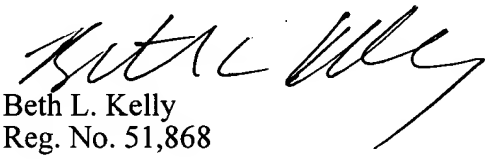
In view of the above arguments, withdrawal of the rejections for alleged lack of enablement is respectfully requested.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance and an action to that end is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 415-576-0200.

Respectfully submitted,



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Blockade of Wnt-5A/Frizzled 5 Signaling Inhibits Rheumatoid Synoviocyte Activation

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Objective. **It is not understood why cultured fibroblast-like synoviocytes (FLS) from patients with rheumatoid arthritis (RA) often display a persistently activated phenotype, despite removal from an inflammatory environment. Previously, we found that these FLS expressed high levels of both Wnt-5A and Frizzled 5 (Fz5), a receptor–ligand pair implicated in both limb bud and bone marrow stem cell development. The objective of the present experiments was to determine whether Wnt-5A/Fz5 signaling contributes to FLS activation.**

Methods. **Wnt-5A expression in FLS was inhibited by transfection with both antisense and dominant negative (dn) vectors. Fz5 signaling was blocked with an antibody to the extracellular domain of the receptor. The effects of these treatments on the expression of the proinflammatory cytokines interleukin-6 (IL-6) and IL-15 and on the expression of receptor activator of nuclear factor κB ligand (RANKL) were assessed by reverse transcriptase–polymerase chain reaction and immunoblotting.**

Results. **Both antisense Wnt-5A and dnWnt-5A vectors, but not empty vector, diminished IL-6 and IL-15 expression in RA FLS. Anti-Fz5 antibody exerted similar effects and also reduced RANKL expression.**

Conclusion. **Wnt-5A/Fz5 signaling may contribute to the activated state of FLS in RA. Receptor antagonists of Fz5 should be considered for the treatment of refractory synovitis.**

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The normal synovium contains both bone marrow–derived macrophages and mesenchymal fibroblast-like synoviocytes (FLS). In longstanding rheumatoid arthritis (RA), the FLS in the synovial lining and underlying connective tissue proliferate to form a pannus, which destroys articular cartilage (1–4). Inflammatory stimuli, especially tumor necrosis factor α (TNFα) and interleukin-1 (IL-1), are potent activators of FLS, and can induce them to produce other cytokines, chemokines, proteolytic enzymes, and inflammatory mediators (5–8). However, rheumatoid FLS activation sometimes continues despite antiinflammatory therapy. Moreover, FLS that are propagated in vitro, without exposure to proinflammatory cytokines, may remain in an activated state.

We have considered the possibility that various members of the Wnt and Frizzled (Fz) families, which control tissue patterning and cell fate determination during embryogenesis, may contribute to autonomous FLS activation. At least 20 Wnt genes and ≥12 Fz genes have been identified. The Wnt proteins are secreted glycoproteins that bind to the cell surface or the extracellular matrix, and thus, probably act locally in an autocrine or paracrine manner (9–12). The Fz proteins resemble typical G-coupled proteins that function as Wnt receptors (13–15). The exact specificities of the Wnt/Fz ligand–receptor pairs have been difficult to determine because some Wnt proteins can interact with multiple Fz receptors. However, there is evidence that Wnt-5A/Fz5 can function as a ligand–receptor pair (16).

The Wnt and Fz family proteins have been shown to cause both cell proliferation (9,17–20) and cell activation (20,21–24). Wnt/Fz-mediated cell proliferation has usually involved nuclear translocation of the multifunctional protein β-catenin, and cell activation in some cases has been shown to involve protein kinase C (PKC) and calmodulin (CaM) kinase II intermediates. The outcome of Wnt/Fz-mediated signaling in a particular cell type is probably determined by the relative stoichi-

EXHIBIT A

ometry of the Wnt and Fz proteins expressed by that cell (22). Wnt/Fz signaling pathways that influence cell activation might be expected to promote cytokine production.

Previously, we reported that activated FLS from RA patients had increased levels of both Wnt-5A and Fz5. Moreover, transfection of normal FLS with a Wnt-5A expression vector stimulated the synthesis of IL-6 and IL-15 (23). The data were consistent with an association between autocrine or paracrine Wnt-5A/Fz5 signaling and FLS activation. However, to support the hypothesis, one would have to show that interference with Wnt-5A/Fz5 signaling in unmanipulated FLS from RA patients could alter their pattern of cytokine production.

As an initial approach to this problem, we used 3 strategies. Cultured FLS were transfected with an antisense expression vector to Wnt-5A, a dominant negative (dn) Wnt-5A expression vector, or an antibody to the extracellular domain of Fz5. To varying degrees, all 3 treatments inhibited FLS activation.

MATERIALS AND METHODS

FLS collection and processing. Primary cultures of RA FLS were obtained from the University of California San Diego core facility directed by Dr. Gary Firestein. The cultures were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gibco) for 7–8 passages.

Construction of antisense and dnWnt-5A constructs and glutathione S transferase–human Fz5 (GST–hFz5) construct. We had previously cloned Wnt-5A into the pcDNA(+) expression vector (Invitrogen, San Diego, CA) (23). The antisense Wnt-5A was made by subcloning the full-length Wnt-5A from the Wnt-5A expression vector in the opposite orientation into pcDNA(−) (Invitrogen), at the *Eco* RV and *Eco* RI sites. The dnWnt-5A was produced by 1) aligning the sequence of human Wnt-5A with the human and *Xenopus* Wnt-11 sequences, 2) amplifying the region of human Wnt-5A corresponding to the truncated (1–282 amino acid) dn *Xenopus* Wnt-11, using gene-specific primers carrying *Eco* RI and *Eco* RV sites at the flanking ends, and 3) cloning the truncated Wnt-5A into the *Eco* RI and *Eco* RV sites of the mammalian expression vector pcDNA3.1(+) (Invitrogen). Wnt-5A was truncated after the SPDYC peptide sequence. The primers used to amplify the truncated Wnt-5A sequence, using the wild-type Wnt-5A clone as a template, were 5′-AACCTTGAATTCAGTTGCTTTGGGGATGGCTGG-3′ (forward) and 5′-TTGGGATATCTCAGCAGTAGTCAGGGCTGGG-3′ (reverse). The dnWnt-5A construct, with an influenza hemagglutinin (HA) peptide tag at the 3′ end, was made by 1) amplifying the dnWnt-5A sequence using the same forward primer and the reverse primer without the stop codon, 2) cloning the modified Wnt-5A into the *Eco* RI and *Eco* RV sites of pcDNA3.1(+), and 3) cloning the HA tag (23) into the *Eco* RV and *Xho* I sites of the modified dnWnt-5A pcDNA.

The GST–hFz5 fusion construct was made by ligating the Fz5 ectodomain in frame with the GST sequence in the pGEX2T bacterial expression vector. The ectodomain of human Fz5, lacking the secretory signal peptide, was amplified using polymerase chain reaction (PCR) and fused to the carboxyl terminus of the GST sequence. Briefly, the primers 5′-CCGCGGATCCCCGGTCTGCCAGGAAATCACGGTG-3′ and 5′-TCCGGAATTCGGTGGCGAACGTGCGCTCGTCGGC-3′ were used to amplify hFz5 complementary DNA using *Pfu* polymerase (Stratagene, La Jolla, CA). The resulting fragment was digested with *Bam* HI and *Eco* RI and cloned into the pGEX2T bacterial expression vector that was digested with the same restriction enzymes. All vectors were sequenced to confirm their identities.

Cytokine and receptor activator of nuclear factor κB (NF-κB) ligand (RANKL) assays. RNA was extracted from the antisense Wnt-5A–, dnWnt-5A–, and empty vector–transfected RA FLS using RNAzol (Gibco). IL-6, IL-15, G3PDH, RANKL, and fibronectin-specific primers were used for estimating the level of expression of the corresponding messages in the different cell populations by reverse transcriptase (RT)–PCR. The G3PDH and fibronectin genes were used as internal controls to detect nonspecific toxicity. The following list summarizes the primer pairs used for RT-PCR analysis: for IL-6, 5′-CAGATGAGTACAAAAGTCCTGA-3′ (forward) and 5′-CTACATTTGCCGAAGAGCCC-3′ (reverse); for IL-15, 5′-GAGTTACAAGTTATTTCACTTGAG-3′ (forward) and 5′-CAAGAAGTGTTGATGAACATTTGG-3′ (reverse); for G3PDH, 5′-ACCACAGTCCATGCCCATCAC-3′ (forward) and 5′-TCCACCACCCTGTTGCTGTA-3′ (reverse); for RANKL, 5′-GCCAACATTTGCTTTCGACATCA-3′ (forward) and 5′-GGGCTCAATCTATATCTCGAACT-3′ (reverse); and for fibronectin, 5′-GTGTGACCCTCATGAGGCAAC-3′ (forward) and 5′-TACTCTCGGGAATCTTCTCTGT-3′ (reverse).

Protein expression and Western blotting. *Escherichia coli* BL21 cells were transformed with the GST-hFz5 fusion construct and induced overnight with 0.1 m*M* isopropylthiogalactose at room temperature. The cells were then pelleted, lysed in phosphate buffered saline (PBS) in the presence of 2 m*M* phenylmethylsulfonyl fluoride (PMSF) (protease inhibitor), and sonicated for 3–4 minutes on ice. After removing the cell debris by centrifugation, the crude lysate was used for Western blotting with a rabbit anti-Fz5 antiserum and preimmune serum (as a negative control) at a 1:8,000 dilution. The peptide **CPILKESHPLYNKVRTGQVPN**, corresponding to amino acids 198–217 of the human Fz5 ectodomain, was synthesized and injected into rabbits by Zymed (South San Francisco, CA) to generate an antiserum specific for the human Fz5 ectodomain (the bold letters in the peptide are the Fz5 sequence; cysteine [C, at the beginning] was added for raising the antiserum). Preimmune serum was collected from the rabbit before the peptide injection.

For making protein extracts from FLS, the cells were grown to confluence in T25 tissue culture flasks in DMEM with 10% FBS. After removal of the medium and treatment of the cells with trypsin–EDTA, cells from each flask were harvested, washed once with PBS, and disrupted with lysis buffer (20 m*M* Tris HCl, pH 7.5, 500 m*M* NaCl, 1% Triton

X-100, 1 m*M* EDTA, 50 m*M* dithiothreitol, and 2 m*M* PMSF) in a microfuge tube. The lysed mix was spun down, cleared of debris by centrifugation, and assayed for total protein using Bradford's reagent (Bio-Rad, Richmond, CA). Approximately 36 μg of each cell lysate was used for Western blotting analyses. Rabbit anti-Fz5 antiserum was used as a primary antibody, and horseradish peroxidase (HRP)–conjugated goat anti-rabbit IgG (Transduction Laboratories, Lexington, KY) was used as a secondary antibody. This was followed by visualization with a chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, NJ). For analysis of RANKL protein, the primary antibody used was goat anti-human RANKL IgG (Santa Cruz, Biotechnology, Santa Cruz, CA), and the secondary antibody used was HRP-conjugated donkey anti-goat IgG (Jackson ImmunoResearch, Avondale, PA).

Transfection of FLS with Wnt-5A vectors. Lipofectamine (Gibco) was used for transfecting FLS with the antisense Wnt-5A, dnWnt-5A, and empty vector constructs. Briefly, FLS (~2 × 10^5/ml) were plated 1 day before transfection in 6-well tissue culture plates (2 ml/well) and incubated at 37°C in 5% CO_2. Plasmid DNA (~1 μg) was complexed with 6 μl of Lipofectamine in 200 μl of serum-free, antibiotic-free medium in a sterile microfuge tube for 30 minutes, after which 800 μl of medium containing 5% FBS was added. The cells in each well of the 6-well plate were washed with sterile PBS and then incubated with 1 ml of the transfection mix for 7–12 hours. One milliliter of culture medium containing 10% FBS was added to each well after 7–12 hours. The transfection mix was replaced by fresh culture medium containing 10% FBS after 24 hours. From 48 to 72 hours after transfection, cells were incubated in 400 μg/ml of medium containing G418 (Gibco) and maintained in culture for at least 2 passages prior to harvesting.

Quantitation of IL-6 and IL-15 protein expression by enzyme-linked immunosorbent assay (ELISA). ELISAs were performed with supernatants from Fz5 antiserum– and control preimmune serum–treated RA FLS, and with total cell extracts of antisense Wnt-5A–, dnWnt-5A–, and empty vector–transfected RA FLS. About 30 μg of either supernatant protein or total cell extract protein was added to microtiter plates that had been coated previously with 2 μg/ml of either anti-human IL-15 monoclonal antibody (mAb) or anti-human IL-6 mAb. After overnight incubation and washing, ~1 μg/ml of either goat anti-human IL-15 polyclonal antibody (R&D Systems, Minneapolis, MN) or biotin-conjugated rat anti-human IL-6 mAb (PharMingen, San Diego, CA) was added to each well. The secondary antibody used was HRP-conjugated donkey anti-goat IgG (Jackson ImmunoResearch) in the case of the IL-15 ELISA. In the case of the IL-6 ELISA, avidin–HRP and biotin solutions (Vector, Burlingame, CA) were added to each well in dilutions suggested by the manufacturer.

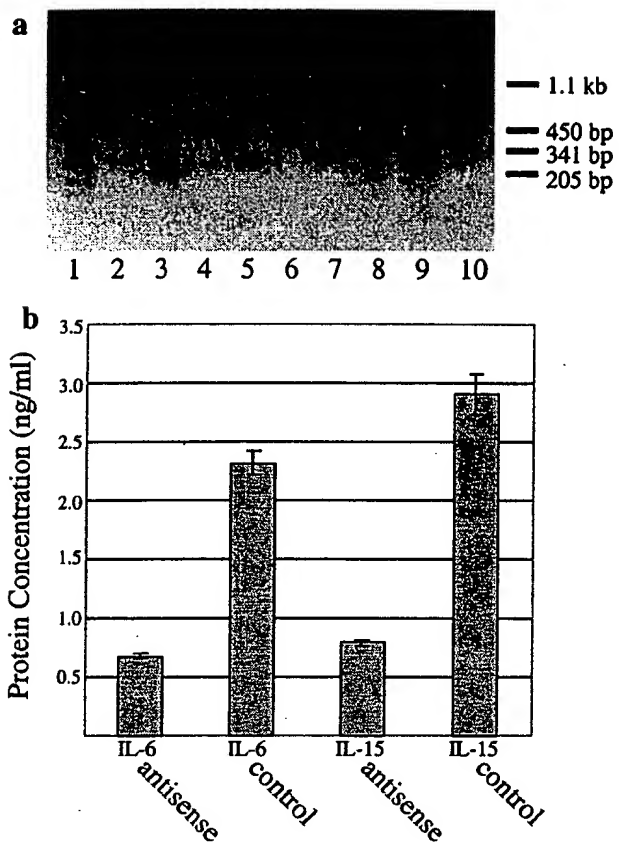

Figure 1. Effect of a Wnt-5A antisense vector on interleukin-6 (IL-6) and IL-15 messenger RNA and protein expression by rheumatoid arthritis fibroblast-like synoviocytes (RA FLS). **A**, Lane 1, PhiX174 DNA standard; lanes 2 and 3, IL-6 gene expression in antisense Wnt-5A– and empty vector–transfected RA FLS, respectively; lanes 4 and 5, IL-15 gene expression in antisense Wnt-5A– and empty vector–transfected RA FLS, respectively; lanes 6 and 7, endogenous Wnt-5A message level in antisense Wnt-5A– and empty vector–transfected RA FLS, respectively; lanes 8 and 9, expression of G3PDH in the same sets of FLS in the same order; lane 10, expression of the antisense Wnt-5A–specific gene product in the antisense Wnt-5A–transfected RA FLS. Results represent an average of 4 experiments performed with FLS from 2 different RA patients. **B**, Results of enzyme-linked immunosorbent assay showing IL-6 and IL-15 levels in antisense Wnt-5A– and empty vector–transfected RA FLS. Results represent the mean ± SEM of 2 different transfections using the same RA FLS.

RESULTS

Expression of IL-6 and IL-15 in RA FLS blocked by both Wnt-5A antisense and dn vectors. Previously, we demonstrated that transfection of normal FLS with a Wnt-5A expression vector increased the expression of IL-6 and IL-15 (23). To determine whether continued Wnt-5A expression was required for the persistent high-level expression of IL-6 and IL-15 in cultured FLS from RA patients, the cells were transfected with either an antisense Wnt-5A construct or a dnWnt-5A construct. In the first set of experiments, RA FLS were transfected separately, either with antisense Wnt-5A cloned in pcDNA or with empty vector pcDNA. After 2 passages

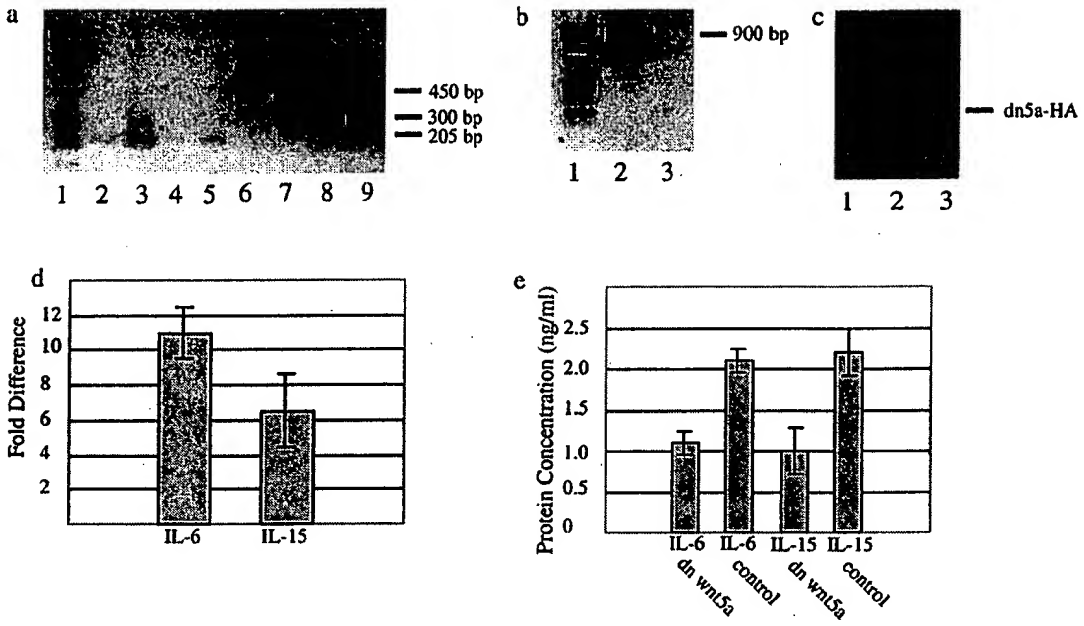

Figure 2. Effect of a dominant negative Wnt-5A (dn wnt5a) vector on IL-6 and IL-15 expression by FLS. **A,** Lane 1, PhiX174 DNA standard; lanes 2 and 3, IL-6 gene expression in dn wnt5a– and empty vector–transfected RA FLS; lanes 4 and 5, IL-15 gene expression in the same sets of RA FLS in the same order; lanes 6 and 7 and lanes 8 and 9, G3PDH and fibronectin expression, respectively, in the same batches of RA FLS. Results represent an average of 4 experiments performed using the same RA FLS in Figure 1. **B,** Lane 1, PhiX174 DNA standard; lane 2, dn wnt5a pcDNA–specific reverse transcriptase–polymerase chain reaction product, which is not present in empty vector–transfected cells (lane 3). **C,** Lanes 1 and 2, dn wnt5a–hemagglutinin (dn5a-HA) protein expression in dn5a-HA–transfected RA FLS; lane 3, dn5a-HA protein is not present in nontransfected cells. **D,** Mean ± SEM magnitude of difference in message intensity levels of IL-6 and IL-15 between empty vector–transfected and dn wnt5a–transfected cells. Results were obtained from 4 different experiments. **E,** Results of enzyme-linked immunosorbent assay showing different levels of IL-6 and IL-15 protein in dn wnt5a– and empty vector–transfected RA FLS. Shown are the mean ± SEM of 3 different transfections using the same RA FLS as in Figure 1. See Figure 1 for other definitions.

in selective medium containing G418, the transfected cells were harvested for RT-PCR analyses of IL-6, IL-15, and the housekeeping gene G3PDH, using gene-specific primers. As shown in Figure 1a, antisense Wnt-5A–transfected cells expressed much less Wnt-5A than empty vector–transfected cells (lanes 6 and 7, respectively), as judged by RT-PCR using primers specific for the untranslated sequence of the endogenous Wnt-5A. The antisense Wnt-5A–transfected cells also expressed much less IL-6 and IL-15 (lanes 2 and 4, respectively) than the empty vector–transfected cells (lanes 3 and 5, respectively). Both, however, expressed similar levels of the housekeeping gene G3PDH (lanes 8 and 9, respectively). Lane 10 shows that the antisense Wnt-5A–transfected cells expressed antisense Wnt-5A, as judged by the results of RT-PCR using antisense Wnt-5A–specific and pcDNA3.1(+)-specific primers. Figure 1b shows that antisense Wnt-5A–transfected RA FLS expressed less IL-6 and IL-15 protein than the empty vector–transfected (control) RA FLS, as measured by ELISA.

Since antisense transfection experiments sometimes produce nonspecific effects on gene expression, it was important to confirm the Wnt-5A antisense results using a different strategy. Previously, Kuhl et al and Du et al demonstrated that a truncated version of Wnt-11 (dnWnt-11), which is functionally very similar to Wnt-5A, abolished some of the effects of Wnt-5A–mediated signaling (24,25). Therefore, we used the same strategy to make a truncated (dn) Wnt-5A construct. In 2 separate experiments, FLS from 2 different RA FLS lines were transfected with either the truncated Wnt-5A construct or empty vector pcDNA. After 2 passages in G418-containing selective medium, the transfected cells were harvested for RT-PCR and Western blot analyses. As shown in Figure 2a, the cells transfected with trun-

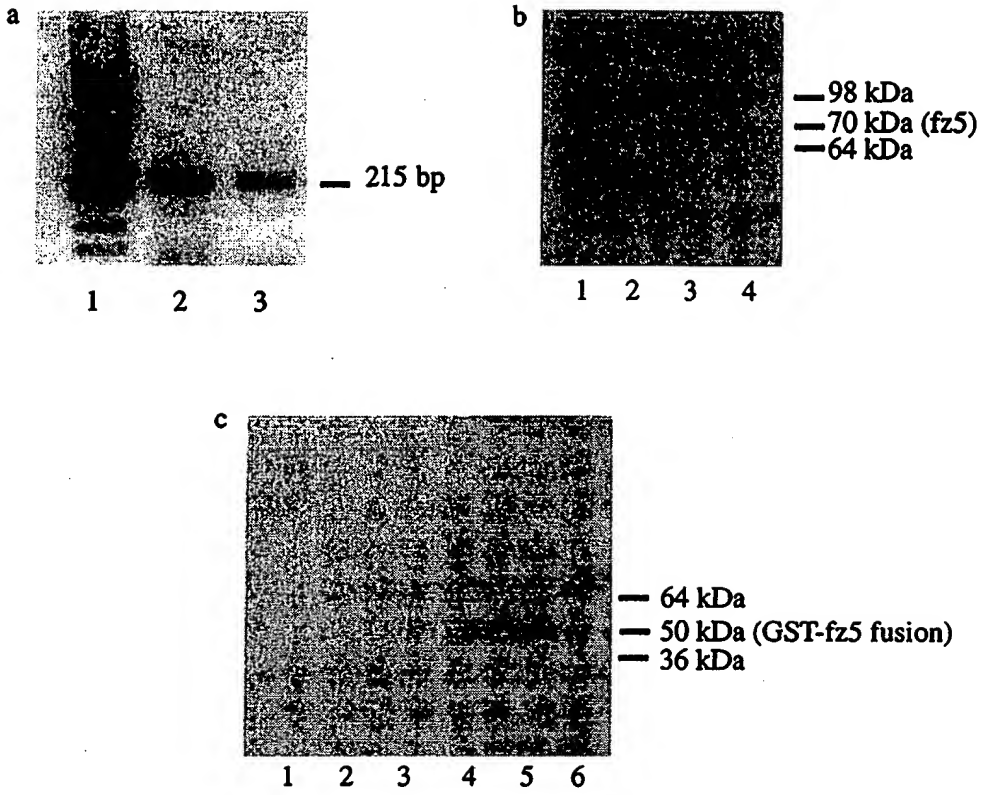

Figure 3. Expression of Frizzled 5 (fz5) in RA FLS. **A,** Lane 1, PhiX174 DNA standard; lanes 2 and 3, fz5-specific gene product in FLS from 2 different RA patients. **B,** Lanes 1 and 2, immunoblots of RA cell extracts probed with preimmune serum; lanes 3 and 4, same RA cell extracts probed with an anti-fz5 antiserum, showing expression of the 70-kd fz5 protein. **C,** Lanes 1 and 2, recombinant glutathione S transferase–fz5 (GST-fz5) fusion protein expressing bacterial lysate probed with preimmune serum; lane 3, an irrelevant GST fusion protein lysate probed with preimmune serum; lanes 4 and 5, 50-kd recombinant GST-fz5 fusion protein band obtained by probing protein blot with the fz5 antiserum; lane 6, irrelevant GST fusion protein treated with the fz5 antiserum. See Figure 1 for other definitions.

cated Wnt-5A (dnWnt-5A) expressed lower levels of both IL-6 and IL-15 (lanes 2 and 4, respectively) than the empty vector–transfected cells (lanes 3 and 5, respectively). Both the dnWnt-5A– and empty vector–transfected FLS, however, expressed relatively similar levels of the housekeeping genes G3PDH (lanes 6 and 7, respectively) and fibronectin (lanes 8 and 9, respectively).

To check transfection efficiencies, we performed RT-PCR analyses on dnWnt-5A–transfected cells using empty vector pcDNA–specific and dnWnt-5A–specific primers and also performed Western blots of dnWnt-5A/HA–transfected cells using anti-HA antibody. Figure 2b shows that dnWnt-5A–transfected cells expressed the dnWnt-5A pcDNA–specific RT-PCR product (lane 2), which was not expressed by the empty vector–transfected cells (lane 3). Figure 2c shows that the dnWnt-5A/HA protein was expressed in RA FLS transfected with the dnWnt-5A/HA construct (lanes 1 and 2), but was not present in nontransfected cells (lane 3). Figure 2d demonstrates the magnitude of difference in IL-6 and IL-15 message levels between empty vector–transfected (control) FLS and dnWnt-5A–transfected FLS. The fold difference using relative band intensities was calculated using Image Analysis Software (Eastman Kodak, Rochester, NY). Figure 2e shows that cell extracts of dnWnt-

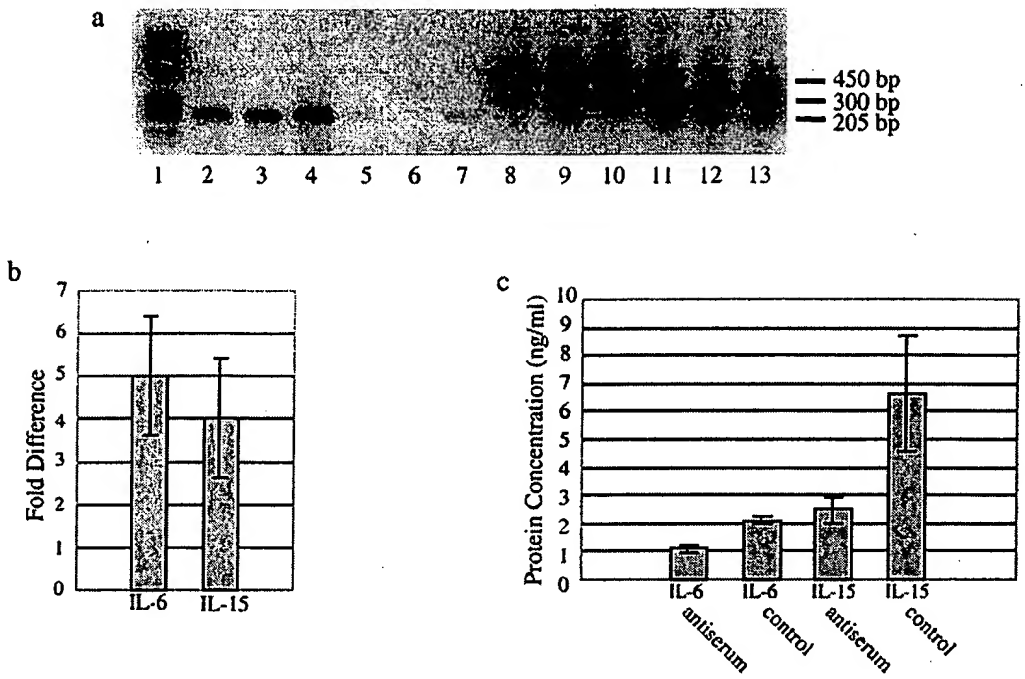

Figure 4. Effect of anti–Frizzled 5 (anti-Fz5) antibody on FLS. **A,** Lane 1, PhiX174 DNA standard; lanes 2 and 3, IL-6 messenger RNA expression in Fz5 antiserum–treated RA FLS; lane 4, IL-6 expression in control preimmune serum–treated RA FLS; lanes 5 and 6, IL-15 expression in Fz5 antiserum–treated RA FLS; lane 7, IL-15 expression in the same RA FLS treated with control preimmune serum; lanes 8–10, G3PDH expression in the same sets of RA FLS in the same order; lanes 11–13, fibronectin expression in the same RA FLS in the same order. **B,** Mean ± SEM magnitude of difference in message intensity levels of IL-6 and IL-15 between preimmune serum– and antiserum-treated RA FLS as determined by Image Analysis Software. **C,** Supernatants from preimmune- and Fz5 antiserum–treated FLS were collected. Levels of IL-6 and IL-15 were determined in triplicate by enzyme-linked immunosorbent assay. Shown are the mean ± SEM IL-6 and IL-15 protein levels. See Figure 1 for other definitions.

5A–transfected RA FLS also expressed lower levels of IL-6 and IL-15 proteins that empty vector–transfected (control) RA FLS, as measured by ELISA.

Expression of Fz5 in RA FLS. Cultures of RA FLS from 2 different patients were harvested at confluence and tested for Fz5 expression by RT-PCR and Western blotting. Figure 3a (lanes 2 and 3) shows that the RA FLS contained the Fz5-specific RT-PCR product, and Figure 3b (lanes 3 and 4) shows that the same FLS expressed the 70-kd Fz5 protein. This Fz5-specific protein band was not reactive with the control preimmune serum from the same animal (Figure 3b, lanes 1 and 2). To ascertain whether the antiserum bound to the Fz5 ectodomain against which it was raised, we expressed the GST-fused Fz5 ectodomain construct in bacteria and performed Western blot analysis on the cell lysate with the anti-Fz5 antiserum. Figure 3c (lanes 4 and 5) shows that the Fz5 antiserum recognized the extracellular region of Fz5. The GST-fused ectodomain of Fz5, when expressed in bacteria, produced an expected 50-kd protein that was not visualized either in lysates of an irrelevant GST fusion protein (lane 6) or in blots probed with preimmune serum (lanes 1, 2, and 3).

Abrogation of RA FLS phenotype by administration of anti-Fz5 antibody. Since Fz5 has been reported to function as a receptor for Wnt-5A, we investigated whether administration of an antibody to the extracellular domain of Fz5 could block paracrine Wnt-5A/Fz5–mediated signaling. In the experiments shown in Figure 4, different RA FLS lines were incubated with a 1:1,000 dilution of anti-Fz5 antibody or preimmune serum in normal growth medium. After 18–40 hours of incubation of 60–80% confluent cells with either antiserum or preimmune serum, the cells were harvested for the

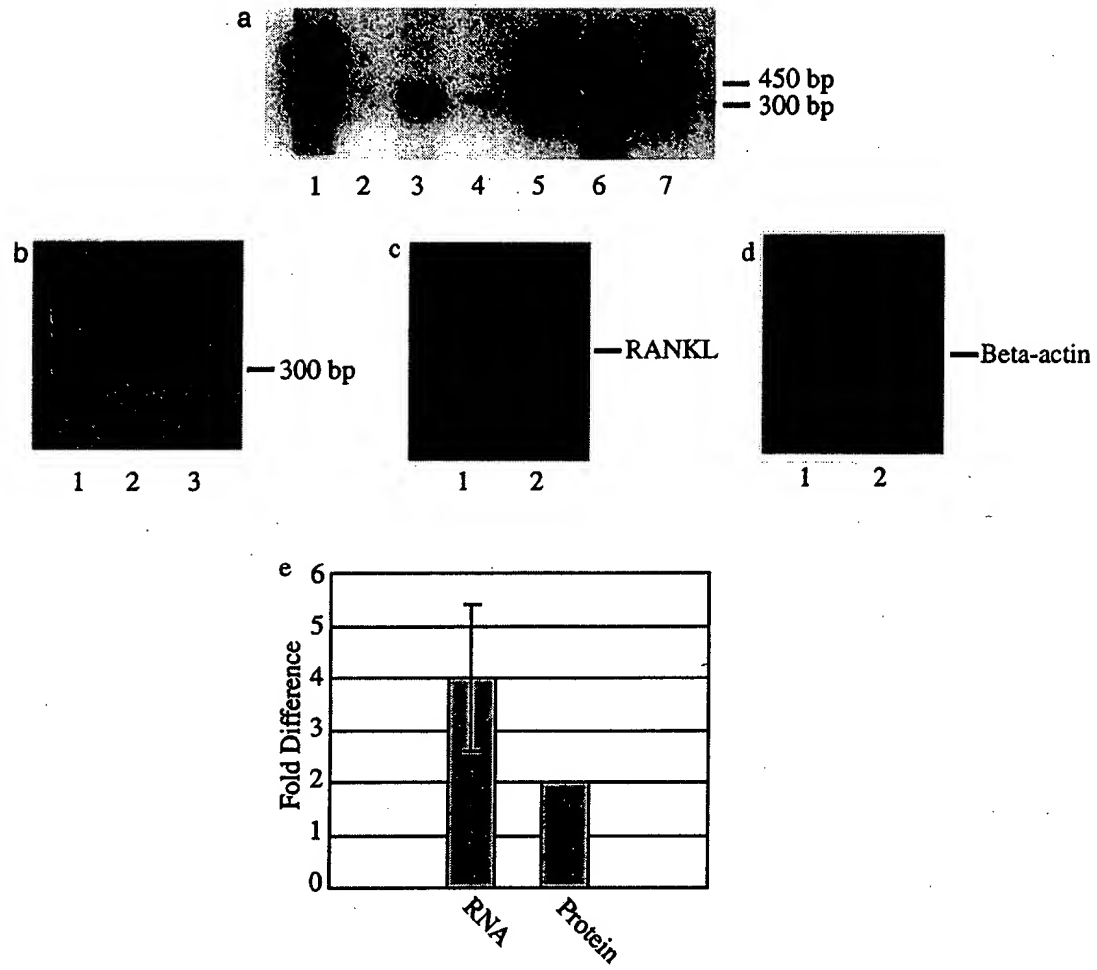

Figure 5. Effect of anti–Frizzled 5 (anti-Fz5) on expression of receptor activator of nuclear factor κB ligand (RANKL). **A,** Lane 1, PhiX174 DNA standard; lanes 2–4, RANKL expression in normal FLS and in 2 batches of RA FLS, respectively; lanes 5–7, G3PDH expression in the same sets of RA FLS in the same order. Results represent an average of 2 different experiments. **B,** Lane 1, PhiX174 DNA standard; lanes 2 and 3, RANKL expression in Fz5 antiserum– and preimmune serum–treated RA FLS, respectively. Results represent an average of 3 different experiments. **C,** Lanes 1 and 2, RANKL protein expression in Fz5 antiserum– and control preimmune serum–treated RA FLS, respectively, by Western blot analysis. **D,** Lanes 1 and 2, β-actin protein expression in Fz5 antiserum– and preimmune serum–treated RA FLS, respectively, by Western blot analysis. Results represent an average of 3 different experiments. **E,** Mean ± SEM magnitude of difference in RNA and protein levels of RANKL between control preimmune serum– and Fz5 antiserum–treated RA FLS. Results are from 3 different experiments. See Figure 1 for other definitions.

analysis of IL message levels by RT-PCR, and cell supernatants were collected for quantitation of IL protein levels by ELISA.

The results of RT-PCR analysis (Figure 4a) showed that, compared with the control preimmune serum, the anti-Fz5 antibody significantly reduced IL-6 and IL-15 messenger RNA (mRNA) levels. Lanes 2 and 3 show IL-6 message levels in antiserum-treated cells, and lane 4 shows IL-6 message level in the preimmune serum–treated cells. Lanes 5 and 6 likewise show IL-15 message level in the same sets of cells in the presence of antiserum, and lane 7 shows IL-15 message level of the cells in the presence of preimmune serum. Lanes 8–10 and 11–13 show G3PDH and fibronectin levels, respectively, in the same sets of cells in the same order. Figure 4b summarizes the differences in the message intensities

of IL-6 and IL-15 between the preimmune serum– and antiserum–treated FLS. The difference in IL-6 and IL-15 mRNA levels between control preimmune– and antiserum-treated cells was reproduced by ELISA of the cell supernatants. As shown in Figure 4c, the antiserum-treated cells produced a mean ± SEM 2.5-fold less IL-15 and ~2-fold less IL-6 than the preimmune serum–treated cells. Both the antiserum- and preimmune serum–treated cells secreted similar levels of the cell matrix protein fibronectin (data not shown), thus ruling out a nonspecific toxic effect of the anti-Fz5 antiserum on the RA FLS. Although the RNA and protein levels of IL-6 and IL-15 were not affected to the same extent by addition of the antiserum, this result is not surprising, since the turnover times of mRNA and protein differ.

Effect of Fz5-mediated signaling on RANKL regulation. To investigate whether Fz5-mediated signaling might play any role in osteoclastogenesis, we studied the effects of anti-Fz5 antibody on the expression of RANKL. It has already been reported that RA FLS express much higher levels of RANKL than normal FLS (26,27). RT-PCR analysis confirmed this observation (Figure 5). Furthermore, treatment of RA FLS with anti-Fz5 antibody resulted in a diminution of RANKL message levels. In Figure 5b, lanes 2 and 3 show RANKL message levels in antiserum- and preimmune serum–treated RA FLS, respectively. Figure 5c shows that RANKL protein was also expressed less in Fz5 antiserum–treated RA FLS (lane 1) than in control preimmune serum–treated RA FLS (lane 2), as measured by Western blot analysis of cell lysates. The levels of the housekeeping protein β-actin were, however, the same in both the antiserum- and preimmune serum–treated RA FLS, as shown in Figure 5d. Figure 5e shows the fold difference in RNA and protein levels of RANKL between the control preimmune– and antiserum-treated RA FLS.

DISCUSSION

In longstanding RA, the FLS acquire an abnormal phenotype characterized by the synthesis of cytokines and metalloproteinases and the up-regulation of cell surface adhesion molecules. These properties may contribute to the destruction of articular cartilage. The activated phenotype of FLS in RA is at least partly autonomous, since it persists after several passages of the cells in tissue culture. Possible explanations for the aggressive characteristics of RA FLS include genetic transformation caused by retroviral infection or somatic mutations, sorting and selection of variants from a preexisting heterogeneous cell population, and migration of new cells from the blood and bone marrow.

The major cytokines found in RA joints (TNFα, IL-1) are potent stimulators of FLS (28,29). They may promote the formation of osteoclast-like cells that can enlarge channels between the bone marrow and the synovial cavity, as has been observed in a murine model of collagen-induced arthritis (30,31). Intense growth stimulation may cause immature mesenchymal cells, which have the characteristics of RA FLS, to replace gradually the normal synovial lining layer of mature FLS.

Considering that Wnt-5A has been shown to influence both limb bud mesenchyme and bone marrow stem cell development, it is conceivable that it also plays a role in chronic joint diseases, which are characterized by tissue destruction and attempted regeneration. Moreover, Wnt-5A has been reported to stimulate the PKC signaling cascade, which potentially can enhance NF-κB activation (32–34), with resultant induction of IL genes such as those for IL-6 and IL-15 (35–37). These IL are readily detectable in the synovia of patients with chronic RA and probably play a role in disease progression. IL-6 is an important mediator of the acute-phase response characteristic of RA (1,5,6). IL-15 shares many activities with IL-2 in stimulating lymphocytes, macrophages, and dendritic cells, and the cytokine also promotes TNF synthesis (38,39).

Recently, IL-15 has been shown to stimulate the differentiation of osteoclast progenitors (40). Bone-resorbing osteoclasts in the inflamed synovium play an important role in the formation of erosions in RA (26,27,30,31,40,41). FLS from RA patients support osteoclast formation in vitro and express RANKL, also known as osteoclast differentiation factor, osteoprotegerin ligand, or TNF-related activation-induced cytokine.

Our results suggest that Wnt-5A/Fz5–mediated signaling by the RA FLS may promote increased production of IL-6, IL-15, and RANKL, and thus contribute to pannus formation, cartilage destruction, and bone erosion. To confirm this supposition, we need to address the effect of Wnt-5A/Fz5–mediated signaling on osteoclastogenesis. We had previously shown that Wnt-5A was overexpressed in RA FLS compared with normal FLS, and that Fz5, a receptor for Wnt-5A, was also highly expressed by RA FLS. We show here that transfection of FLS from RA patients with either an antisense Wnt-5A vector or a dnWnt-5A vector down-regulated

IL-6 and IL-15 expression without altering substantially the expression of housekeeping genes.

The stoichiometric levels of Wnt-5A and Fz5 required to initiate signaling are not clearly known. The exact signaling molecules that mediate Wnt-5A/Fz5 signaling in RA FLS also need to be identified. Our experiments did not address directly whether Wnt-5A/Fz5 signaling in the RA FLS is dependent on PKC, CaM kinase II, glycogen synthase kinase 3β, or β-catenin, which have been shown to be mediators of Wnt/Fz signaling in other systems. However, overexpressed Wnt-5A could plausibly lead to autonomous FLS activation via autocrine and/or paracrine loops involving cell surface Fz5 receptors. Consistent with this notion, treatment of RA FLS with a polyclonal antibody specific for the extracellular domain of Fz5 blocked IL expression at both the RNA and protein levels. RANKL expression by RA FLS was also reduced by treatment with anti-Fz5 antiserum.

The quantitative levels of reduction in IL-6 and IL-15 expression in RA FLS by antisense/dnWnt-5A administration and anti-Fz5 antiserum treatment did not match completely. This finding is not surprising, because the antisense Wnt-5A and dnWnt-5A vectors may not have the same absolute specificities. Due to the high degree of homology between Wnt and Fz family members, and given the diversity of Wnt genes (some of which have not yet been characterized functionally), the vectors conceivably could affect the expression/function of more than one Wnt gene. However, considering that anti-Fz5 antibody exerted effects similar to those of antisense Wnt-5A and dnWnt-5A, and given our earlier data showing that Wnt-5A and Fz are overexpressed in RA FLS, the results document a role for Wnt-5A/Fz5 signaling in RA. Chronic exposure to growth factors and inflammatory mediators in the RA synovium may favor the gradual replacement of normal FLS with immature FLS expressing Wnt-5A and Fz5. Autocrine or paracrine signaling through the Wnt-5A/Fz5 pathway may then enhance the synthesis of IL-6 and IL-15. The cytokines in turn may promote osteoclast differentiation through RANKL up-regulation.

Currently, there are no approved drugs or biologics that target FLS in RA. The Fz proteins resemble typical G protein–coupled receptors, and should be amenable to pharmacologic manipulation. In our experiments, antibody to Fz5 was not toxic to FLS, but blocked their activation. Thus, the Wnt-5A/Fz5 pathway may be an attractive target for the development of new therapeutic agents in RA.

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Wnt5a signaling directly affects cell motility and invasion of metastatic melanoma

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Summary

Gene expression profiling identified human melanoma cells demonstrating increased cell motility and invasiveness. The gene *WNT5A* best determined in vitro invasive behavior. Melanoma cells were transfected with vectors constitutively overexpressing Wnt5a. Consistent changes included actin reorganization and increased cell adhesion. No increase in β-catenin expression or nuclear translocation was observed. There was, however, a dramatic increase in activated PKC. In direct correlation with Wnt5a expression and PKC activation, there was an increase in melanoma cell invasion. Blocking this pathway using antibodies to Frizzled-5, the receptor for Wnt5a, inhibited PKC activity and cellular invasion. Furthermore, Wnt5a expression in human melanoma biopsies directly correlated to increasing tumor grade. These observations support a role for Wnt5a in human melanoma progression.

Introduction

The incidence of cutaneous melanoma is increasing, with an estimate of 1 in 75 people in the United States contracting the disease in the year 2000 (Landis et al., 1999). Although early diagnosis is associated with a high cure rate, due to the highly malignant nature of this disease, 20% of diagnosed patients will die from advanced disease. Unfortunately, it remains difficult to predict a priori which patients are more likely to develop highly invasive disease, as melanoma types are essentially clinically indistinguishable based upon routine histopathologic criteria (Weyers et al., 1999). Therefore, markers of melanoma invasiveness would be of both biological and possibly clinical utility. In a recent study of the authors, gene expression analysis was used to identify a series of genes whose expression differed between cutaneous melanomas with differing invasive phenotypes (Bittner et al., 2000). One of the genes identified as a particularly robust marker of highly aggressive behavior in this study was the gene *WNT5A*.

Wnt5a is a member of the Wnt family of proteins, which are 38–45-kDa secreted cysteine-rich proteins with hydrophobic signal peptides. They have no transmembrane domains and are posttranslationally modified by N-linked glycosylation (Moon et al., 1997). Vertebrate *WNT* genes are expressed in unique but overlapping patterns during gastrulation, and, in the adult, they are expressed in a variety of tissues. Each of these signaling proteins carries information distinct from other family members, and stimulation by multiple family members can produce results different from either single input, implying that family members can influence the interpretation of each others' signals. Such interactions provide the possibility of considerable subtlety and complexity in Wnt signaling. Secreted Wnts associate with cell surfaces and the extracellular matrix, and many are shown to closely associate with the Frizzled family of receptors (Yang-Snyder et al., 1996). Wnt signaling has been shown to be important not only in development, but also in tumorigenesis. For example, Wnt1 can signal via Frizzled to hyperphosphorylate and activate the disheveled gene, resulting in the inhibition of GSK3-β activity and the subsequent stabilization of its target β-catenin (Miller et al., 1999). β-catenin can then accumulate in the nucleus, altering chromatin structure, which results in differential gene expression (Sharpe et al., 2001). As β-catenin is also known to interact with E-cadherin, a cell-cell adhesion molecule, this further suggests a role for *WNT* genes in modulating cell-cell interactions (Moon et al., 1993).

In tumorigenesis, the abnormal expression of the Wnt family members can be divided into three distinct types of transforming ability. The highly transforming members of this family include

SIGNIFICANCE

Comparative studies of metastatic versus nonmetastatic cells have identified several differences in morphology and behavior between these phenotypes. It is more difficult to demonstrate how cells shift from one phenotype to the other. Many hypotheses exist, from random accrual of the necessary changes during periods of chromosomal instability to dysregulation of normal cell-to-cell signaling interactions. Here, we describe how increasing the level of a single signaling component can evoke a complex change in cell phenotype reflecting the properties expected with a shift to a highly metastatic cancer cell phenotype. With the in vitro observations directly correlating with results from human melanoma tumor biopsy samples, this paper provides support for the notion that the Wnt5a pathway is of possible clinical significance in melanoma.

EXHIBIT B

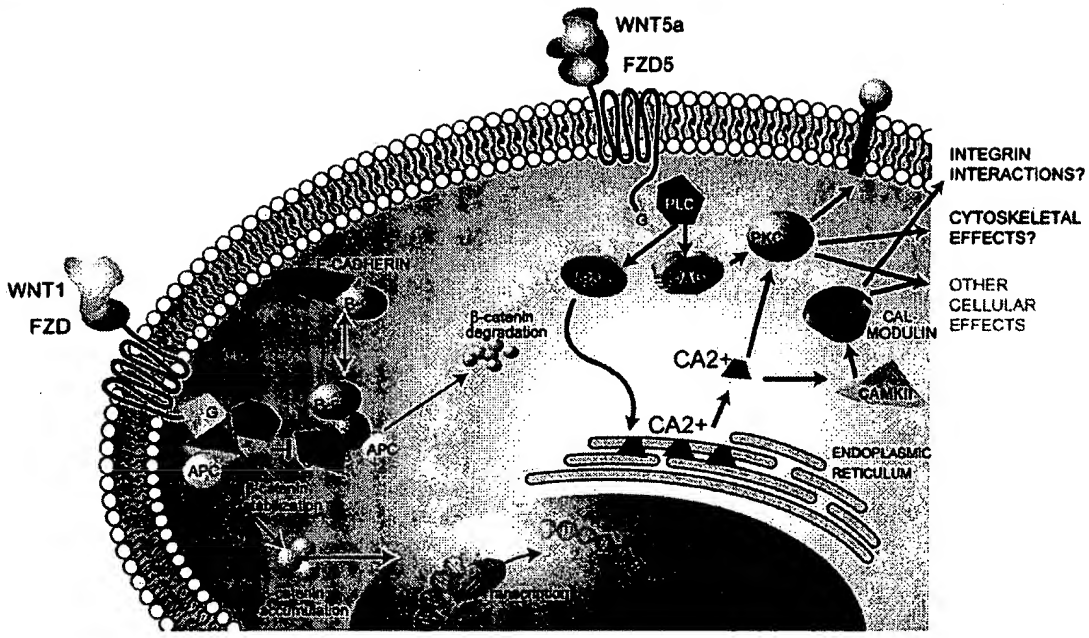

Figure 1. The Wnt signaling pathways

Wnt1 signals via Frizzled to activate the disheveled protein, resulting in the stabilization of β-catenin and its subsequent translocation. Wnt5a, however, signals via Frizzled to activate PLC causing phospholipid turnover in the membrane, releasing calcium from intracellular stores and increasing PKC activity.

Wnt1, Wnt3a, and Wnt7a. The intermediately transforming members include Wnt2, Wnt5b, and Wnt7b, and the nontransformers include Wnt4, Wnt5a, and Wnt7b (Jue et al., 1992; Wong et al., 1994). In fact, Wnt5a can interact in a cell nonautonomous manner to block Wnt1 signaling during development as well as during transformation. For example, the expression of Wnt5a can cause a failure of Wnt1 to duplicate the embryonic axis during *Xenopus* development (Torres et al., 1996). In tumorigenesis, mouse C57MG mammary cell transformation by an antisense Wnt5a mimics Wnt1-mediated transformation (Olson and Gibo, 1998). Furthermore, ectopic expression of Wnt5a in uroepithelial cancer reverts tumorigenesis (Olson et al., 1997). Thus, while the signal transduction pathways of Wnt1 and Wnt5a remain very distinct from each other (Figure 1), Wnt5a appears to be able to affect the results of Wnt1 signaling.

It has been hypothesized that Wnt5a is an important regulator of cell growth and differentiation and that loss of expression may in some cases lead to transformation. However, there are several other lines of evidence that indicate that continued or increased Wnt5a expression is indeed important in cancer. Wnt5a is upregulated in cancers of the lung, breast, and prostate and is downregulated in pancreatic cancer (Cmogorac-Jurcevic et al., 2001; Iozzo et al., 1995; Lejeune et al., 1995). However, in cancers of the bladder, there is no change in Wnt5a expression, implying that its loss is not a necessary precursor to tumorigenesis in general (Bui et al., 1998). The overexpression of Wnt5a in all of these tumors is not a result of gene amplification or rearrangement, suggesting that the level of Wnt5a is being modulated in these cases by some further regulatory apparatus. The present study aims to focus on the ability of Wnt5a to alter phenotypes leading to increased invasiveness of melanoma cells and to examine the expression patterns of this protein in human melanoma.

Results

Overexpression of Wnt5a changes the morphology of melanoma cells

UACC 1273 melanoma cells (derived from an axillary lymph node in a 54-year-old male patient) were selected based on their low Wnt5a expression and low in vitro invasion (Bittner et al., 2000). Cells were transfected with plasmid vectors capable of constitutively expressing Wnt5a. Clones were selected in G418 and were examined for Wnt5a expression using both real-time PCR and immunohistochemistry (Figure 2). Clones that expressed Wnt5a at significantly increased levels relative to the parent are designated as 1273 1-3, 1273 4-3, and 1273 4-7. Clones designated 1273 1-1 and 1273 EV (empty vector) do not express Wnt5a at levels significantly different than the parental line. Although there are no great differences in cell proliferation or apoptosis, the shape of cells expressing higher levels of Wnt5a is drastically different from the parental cell shape. The parental cells are compact, thick, and roughly triangular, with few points of contact with the substrate and few extended processes. The transfected daughter cells are thin and spreading, with irregular shapes, many points of contact with the substrate, and numerous extended processes (Figure 3A). When stained with fluorescent phalloidin in this study, actin clustered in long filaments along the edges of the high-Wnt5a-expressing cells, but not in the low-Wnt5a-expressing cells (Figure 3B), suggesting that actin reorganization was a consequence of in-

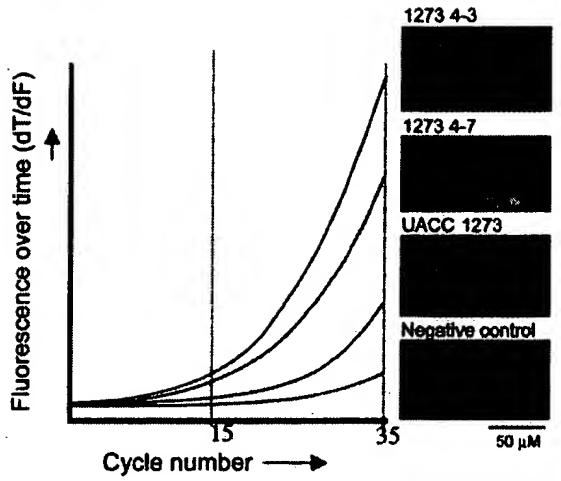

Figure 2. Wnt5a expression in transfected melanoma cells

UACC 1273 melanoma cells were transfected with Wnt5a under control of the CMV promoter, using the Gateway cloning system from Invitrogen. Cells were assayed for Wnt5a expression using both real-time PCR for the Wnt5a transcript and immunohistochemistry for the Wnt5a protein.

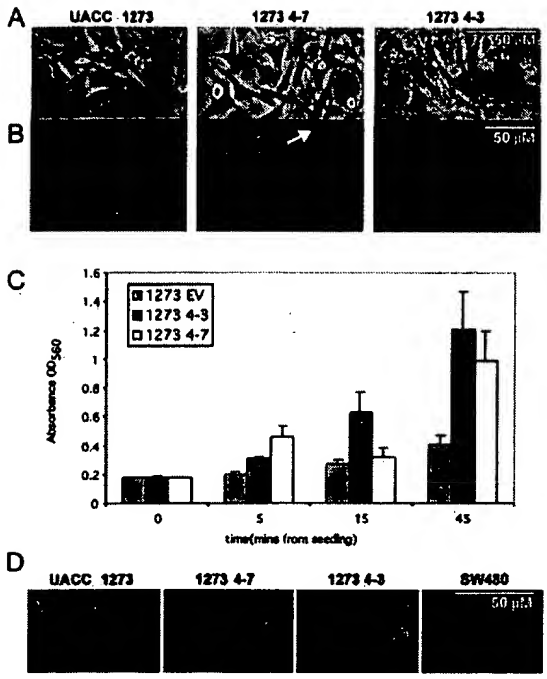

Figure 3. Effects of Wnt5a transfection on melanoma cells

A: The morphological effects of Wnt5a transfection. The transfection of Wnt5a into UACC 1273 cells results in a change from compact cobblestone-shaped cells to irregularly shaped cells with many protruding processes. **B:** The effects of Wnt5a transfection on actin rearrangement. Transfection of Wnt5a into UACC 1273 cells results in a polarization of actin at the edges of the cells. There is also evidence of actin ruffling, at the end of the elongated cells (arrow), perhaps indicative of increased invasion. **C:** Wnt5a increases cell adhesion. A total of 50,000 cells were seeded for the indicated times, and nonadherent cells were rinsed off. Adherent cells were stained with crystal violet and were analyzed spectrophotometrically. Cells transfected with Wnt5a adhered much sooner than cells transfected with an empty vector control. **D:** Wnt5a does not affect β-catenin expression and translocation. Whether cells were transfected with an empty vector control or Wnt5a, β-catenin translocation to the nucleus could not be observed; staining remained pericellular. SW480 colon cancer cells, which have a mutant APC gene resulting in overexpression of β-catenin, were used as a positive control and demonstrated abundant staining for nuclear β-catenin.

creasing Wnt5a in these cells. Actin also clustered at the edges of the cell processes, demonstrating what appeared to be membrane ruffling, which is potentially indicative of increased cell motility (arrows, Figure 3B). Wnt5a-transformed cells also showed more resistance to trypsinization than the parental or vector control cells, presumably indicating increased adhesion to the extracellular matrix (ECM). In order to confirm this observation, cell adhesion assays were performed. Cells transfected with Wnt5a (1273 4-3 and 1273 4-7) adhered more readily than empty vector controls (Figure 3C).

Overexpression of Wnt5a in melanoma cells has no effect on β-catenin expression or translocation but increases the activity of protein kinase C

β-catenin mutations are thought to play some role in melanoma (Rubinfeld et al., 1997), and β-catenin is important in cell adhesion. Based on the observation that Wnt5a-transfected cells demonstrated changes in cell adhesion, we examined the effect of Wnt5a on β-catenin in our transfectants. However, there was no change in β-catenin expression, and expression was localized to the periplasmic membrane and cell-cell junctions, presumably in conjunction with E-cadherin (Figure 3D).

A signal transduction pathway known to be downstream of Wnt5a is the calcium pathway, which results in the activation of protein kinase C (PKC) (Kuhl et al., 2000). The PKC pathway has been shown to be important in melanoma and other cancers, and its action is often associated with changes in the cytoskeleton, cell adhesion, and motility (Szalay et al., 2001; Timar et al., 1997), leading to the expectation that the phenotypic changes observed upon increased Wnt5a expression would result in observable increases in activation of PKC by phosphorylation. When the level of activated PKC was examined, it was dramatically increased only in the Wnt5a-transfected cell lines that showed significantly increased Wnt5a expression (1273 1-3, 1273 4-3 and 1273 4-7, Figure 4A). In addition, specific isoforms of PKC were activated in the transfectants compared to the controls (Figure 4B). The two isoforms that were most significantly increased in these transfectants were isoforms μ and α/βII, which are thought to be associated with cytoskeletal organization and invasion, respectively (Bowden et al., 1999; Liu et al., 1994; Timar et al., 1996). The expression of the various PKC isoforms in the Wnt5a transfectants and their association with different aspects of cell growth and motility are summarized in Figure 4B.

Wnt5a leads to an increase in motility and invasion

Based on the observations that cell-ECM adhesion was increased and that isoforms of PKC known to be important in cytoskeletal reorganization and motility were activated by Wnt5a transfection, we examined the in vitro motility of these cells. We observed a striking difference in the rates of motility of the

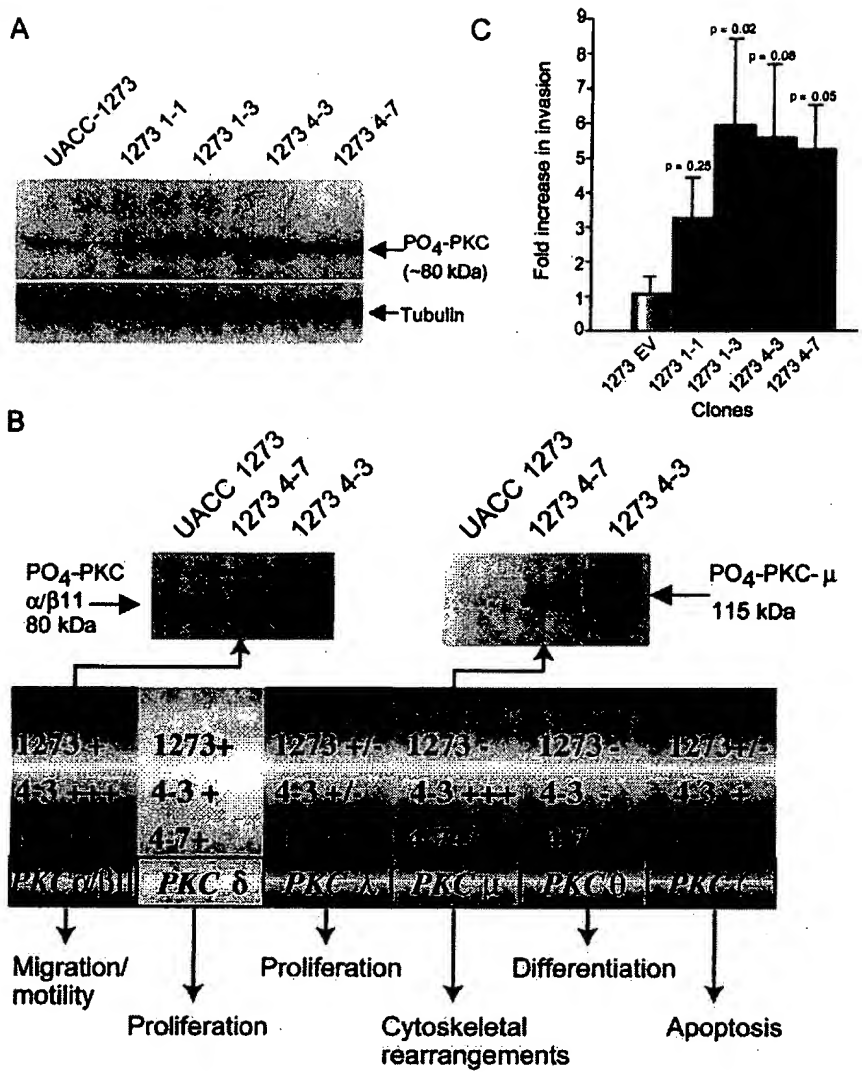

Figure 4. Wnt5a and the PKC pathway

A: Wnt5a increases PKC activity. UACC 1273 and clones transfected with Wnt5a were ordered on a gel from low to high Wnt5a expression and were then probed with an antibody to phospho-PKC, to determine PKC activity. PKC activity was strongly correlated to Wnt5a expression, as determined by both Western analysis and real-time PCR. **B:** PKC isoforms and cellular effects of their activation. Wnt5a transfectants were analyzed for their expression of the various PKC isoforms. Shown here are two example Westerns for differences in activation of PKC α/β II and PKCμ (PKD) upon Wnt5a transfection. The table beneath summarizes the changes in activity for the various PKC isoforms in each transfectant, and the arrows indicate in which cellular functions the various isoforms have been implicated. **C:** Wnt5a transfection increases the invasive ability of melanoma cells. Wnt5a stable transfectants were assayed for invasion in a Boyden chamber assay as described above. The clones were up to 6-fold more invasive than the empty vector-transfected cells. The cell lines shown here are ordered according to increasing PKC activation and Wnt5a expression, and the results are the sum total of four separate assays in which each condition was performed in triplicate. p values for all the assays were calculated using ANOVA analysis. Interestingly, the clone 1273 1-1, which did not significantly increase PKC activation, was not statistically significant in its upregulation of invasion (p = 0.25) as compared to the other clones (p values ranged between 0.02 and 0.08).

transfected cells expressing Wnt5a, correlating to increased motility in endogenously high Wnt5a-expressing cells that were identified by microarray analysis in the initial study by Bittner et al. (Bittner et al., 2000). In order to approximate in vitro invasiveness, we also performed Boyden chamber invasion assays, which mimic the three-step hypothesis of invasion-adhesion, proteolytic dissolution of the extracellular matrix, and migration (Albini, 1998). The cells are placed on a filter coated with reconstituted basement membrane, and migration ("invasion") through the filter is analyzed by fluorimetry. Using this assay, Wnt5a-transfected melanoma cells were up to four times more invasive than empty vector controls or the original low-Wnt5a parental cells, with p values between 0.02 and 0.08 (Figure 4C). The invasion assay results correlate directly with the PKC phosphorylation assay results. Those clones showing increased Wnt5a expression and PKC phosphorylation show significant increases in invasiveness (p values of 0.02–0.08), while clone 1-1, which shows neither increased Wnt5a expression nor increased PKC phosphorylation, does not show a significant increase in invasiveness (p value of 0.25). It is also of interest to note that, after a certain level of PKC activity was attained, there was no further effect on invasion, suggesting a threshold effect for this mechanism. This idea of a threshold effect is supported by the observation that, when Wnt5a was transfected into endogenously high-Wnt5a-expressing melanoma cells, even in clones that did demonstrate increases in PKC activity, invasion was already at a maximum level (Figure S1, see the Supplementary Material available with this article online at http://www.cancercell.org/cgi/content/full/1/3/279/DC1).

Disruption of the Wnt5a/Frizzled-5 pathway results in an inhibition of PKC activation and reduced invasiveness of melanoma cells

As increasing Wnt5a appeared to be positively correlated to motility and invasiveness, it was logical to determine whether the inhibition of Wnt5a signaling would result in the inhibition of in vitro motility and invasion. Because Wnt5a binds to the receptor Frizzled-5, and by this interaction can exert its signaling

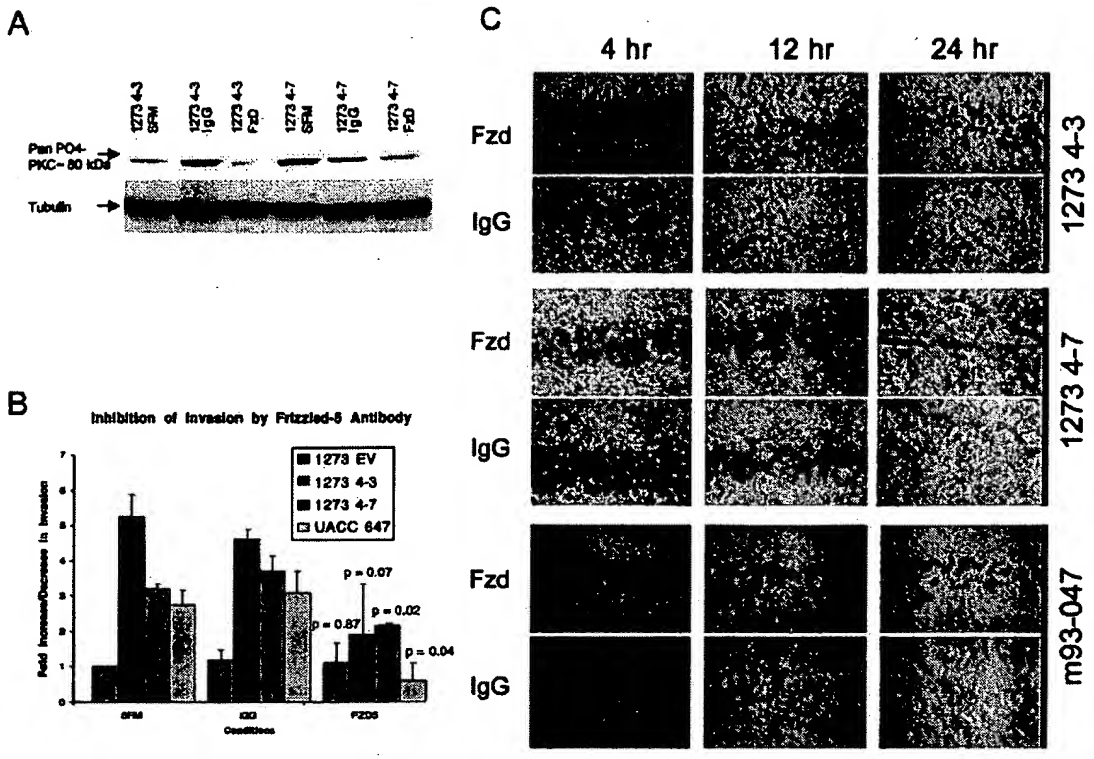

Figure 5. Effects of inhibiting the Frizzled-5 receptor

A: Inhibition of the Fzd5 receptor results in a decrease in PKC activation. The Wnt5a transfectants were treated with a functional antibody to the receptor. This resulted in a decrease in PKC activation in the antibody-treated cells, but not the IgG-treated cells. **B:** Inhibition of the Frizzled-5 receptor results in an inhibition in invasion. Wnt5a transfectants, as well as one high endogenous expresser of Wnt, UACC 647, were pretreated with the antibody to the Frizzled receptor or with IgG for 16 hr prior to the invasion assays. Fzd5-treated cells showed a drastic decrease in invasion as compared to IgG-treated or untreated cells. No difference could be observed in the barely invading vector control cells. p values are shown for treated cells as compared to IgG controls. **C:** Inhibition of the Frizzled-5 receptor results in an inhibition in invasion in a scratch assay. Wnt5a transfectants, as well as cell lines endogenously high in Wnt5a (M93-047 is shown here as an example; for additional lines, see the Supplementary Material at http://www.cancercell.org/cgi/content/full/1/3/279/DC1) were treated with antibody to the Fzd5 receptor in fibronectin-coated chambers. As with the Boyden chamber assays, Fzd5-treated cells showed a drastic decrease in invasion as compared to IgG-treated or untreated cells. Note the still evident scratch in the Fzd5-treated cells even after 24 hr.

effects (Ishikawa et al., 2001; Sen et al., 2000), we first examined our cells for the presence of Frizzled-5. Using real-time RT-PCR, we found that Frizzled-5 was indeed present in these cells, and its expression remained unchanged by artificially increased Wnt5a expression. An antibody against Frizzled-5 has been shown to disrupt the Wnt5a/Fzd5 interaction (Sen et al., 2001); we obtained this antibody, purified the IgG, dialyzed the antibody, tested it by Western analysis, and treated cells with either the antibody or purified dialyzed IgG from the preimmune serum at 100 μg/mL (antibody was replaced every 12 hr for the duration of the assays). In contrast to controls, cells treated with antibody to Frizzled-5 showed a marked decrease in the level of phospho-PKC after 16–24 hr of treatment (Figure 5A). In addition, the in vitro invasion or motility rates of these cells were significantly decreased in the Boyden chamber invasion assay (Figure 5B), in which invasion was inhibited by up to 2.4-fold in the clone 1273 4-3 ($p = 0.02$) and up to 1.7-fold in the clone 1273 4-7 ($p = 0.08$). In an endogenously high-Wnt5a-expressing cell line, UACC 647 Frizzled-5 antibody inhibited the invasion of these cells by over 2-fold ($p = 0.035$). This inhibition was also observed in scratch assays. First, using a scratch assay, cells were seeded on a chamber slide, coated with either fibronectin or collagen, grown to confluence, and then "scratched" with a sterile pipette tip. The time that it takes for each cell line to invade the scratch and begin to fill it can be used as a measure of motility (Bittner et al., 2000). These assays were performed both on the transfectants and on cell lines endogenously high in Wnt5a expression, as determined by microarray analysis. Transfectants 1273 4-3 and 1273 4-7 were significantly inhibited in their invasion even as early as 12 hr (Figure 5C). Three cell lines endogenously high in Wnt5a expression (UACC 930, UACC 647, and M93-047) demonstrated similar rates of inhibition by the Frizzled-5 antibody, and the cell line M93-047 is shown here as an example (Figure 5C). Other cell lines, UACC 647 and UACC 930, are shown in Figure S2 contained in the Supplementary Material available with this article online (http://www.cancercell.org/cgi/content/full/1/3/279/DC1). These experiments clearly show that both the activity level of the presumptive Wnt5a signaling pathway and the activity level of the invasive phenotype are increased by increasing the level of the Wnt5a ligand and are decreased by desensitization of the Wnt5a receptor to the presence of its ligand.

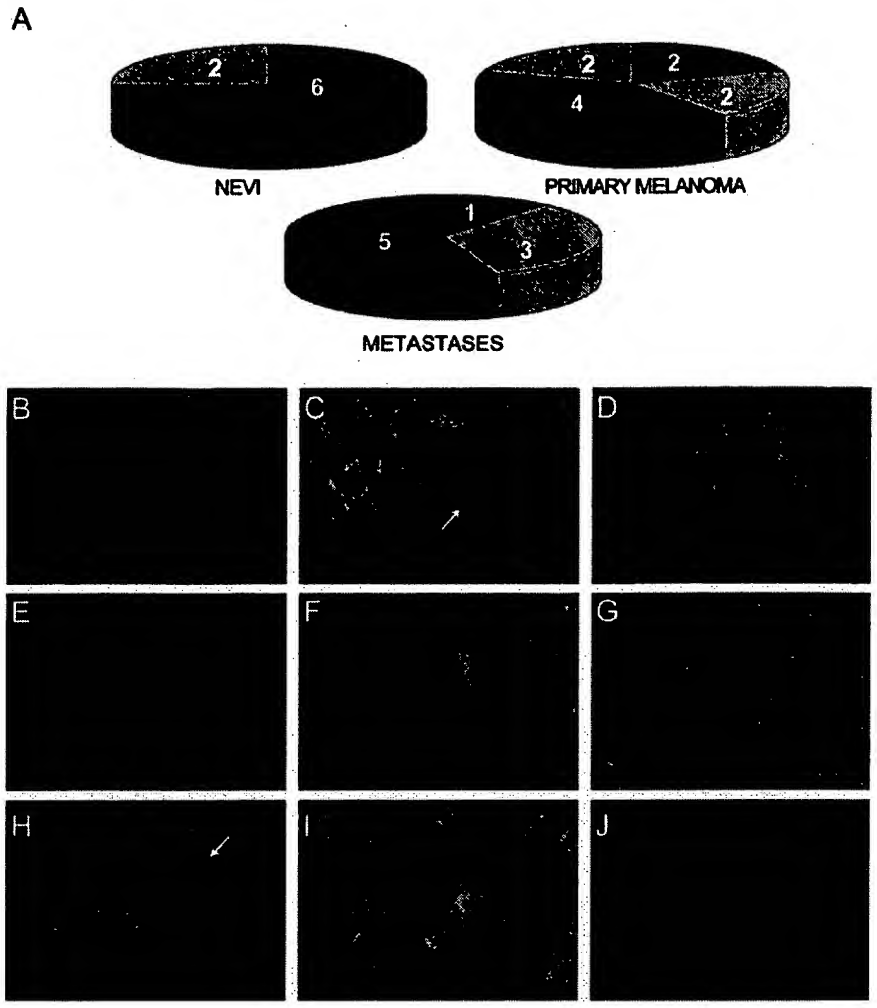

Figure 6. Wnt5a staining of human melanoma

A: Distribution of Wnt5a positivity among tumor types. Wnt5a negativity is represented by blue, moderate positivity for Wnt5a is represented by orange, and strong positivity for Wnt5a is represented by red. Where tumors stained mostly negative with a few foci of positivity, areas are shaded gray. Most benign tumors (NEVI) stain negative for Wnt5a, with two staining focally positive. Wnt5a staining is heterogeneously distributed among primary melanomas (PRIMARY MELANOMA), but most metastatic tumors stain positive for Wnt5a (METASTASES), with one exception. Interestingly, none of the metastases observed thus far stained focally positive for Wnt5a. **B–J:** Wnt5a protein expression in human tissue. Tissues were stained with the antibody to Wnt5a, were secondary-stained with Cy-5 (red staining), and were counterstained with DAPI (blue staining). The distribution of the protein among tissue and cell types was observed. Wnt5a is not expressed in the majority of nevi (**B**), except in two out of the eight samples, in which there is focal positivity for Wnt5a (**C**), although most of the sample and the epidermis stains negative ([**C**], arrow). In primary melanomas, the staining was more heterogenous, where some tumors stained only focally positive for Wnt5a (**D**) and others stained strongly throughout the tumor (**E**). In one primary melanoma, where cells were in vertical growth phase, cells at the leading edge of invasion into the extracellular matrix were strongly positive for Wnt5a (**F**). Eight out of nine metastases exhibited positive staining (**G**), which was specific to the melanoma cells and not to surrounding cell types such as lymphocytes ([**H**], arrow). Upon increased magnification of cells exhibiting giant cell morphology, a phenotype that is highly associated with malignancy, Wnt5a staining is strongly positive, as it usually is in these cases (**I**). In this particular biopsy, the time to metastasis was 17 months, and the patient did not survive. Interestingly, in a histopathologically similar tumor, there was very focal Wnt5a positivity, with mostly negative staining through out the tumor (**J**). This is an unusual observation for these types of tumors, as most are strongly positive for Wnt5a; the patient did not present with metastases for several years and is still alive today.

High Wnt5a expression in tumors is prevalent in cells of particular histological types that are more frequently observed in higher grades of tumor

It has long been appreciated that the fraction of cells in a given tumor that are actively moving to a site distant from the primary tumor is small. An early study investigated the rate at which a transplanted fibrosarcoma, a tumor known for shedding cells into the circulatory system, could place cells into the bloodstream. It was observed that a tumor having a volume of 1.5–2 ml released approximately 10^4 cells to the blood circulation per day (Liotta et al., 1974). Given the premise that a mere 1% of cells within a high-grade primary tumor exhibit a metastatic phenotype, it is reasonable to assume that these cells would constitute such a low percentage of the total mass of the tumor that any RNA expression pattern derived from this metastatic cell population would most likely be diluted to undetectability by the gene expression pattern of the more abundant, nonmetastatic portion of the tumor. What was seen in the previous profiling study (Bittner et al., 2000) is consistent with this view. High Wnt5a expression was not observed in any of the tumor biopsies examined in that paper. High Wnt5a expression was only observed in some clonal cell lines derived from melanomas.

To further test the expectation that high Wnt5a expression would be observed only in the limited fraction of cells in tumors that were actively invasive, tissues of varying stages of melanomas from 27 patients were analyzed for Wnt5a expression in a blind comparison. Paraffin-embedded tumors were sectioned, and serial sections were mounted and subjected to either standard histochemical staining with hematoxylin and eosin (H&E) or to immunohistochemical staining using a biotinylated Wnt5a antibody and a streptavidin-Cy5 secondary antibody with a DAPI nuclear counterstain. H&E-stained sections were used to choose regions of the tumor where the cells displayed features associated with more aggressive tumor behavior (high-grade

Table 1. Distribution of Wnt5a positivity and relation to pathology, outcome, and survival data

Pathology	Wnt5a	Time to development of metastasis	Survival status
Nevus	negative	none	living-no tumor
Nevus	negative	none	living-no tumor
Nevus	negative	none	living-no tumor
Nevus	negative	none	living-no tumor
Nevus	negative	none	living-no tumor
Nevus	negative	none	living-no tumor
High-risk nevus	focally positive	none	living-no tumor
High-risk nevus	focally positive-strong	lost to follow-up	unknown
Primary melanoma	negative	none in 3 years	living-no tumor
Primary melanoma	positive	2 years	unknown
Primary melanoma	focally positive-strong	16 months	deceased
Epidermis melanoma in situ	strong	local recurrence	living-no tumor
Thin invasive melanoma	strong	22 months	living-with tumor
Primary melanoma	focally positive	9 years	living-with tumor
Primary melanoma, stem cell cytology	negative	unknown	unknown
Primary melanoma	positive	local recurrence	living-no tumor
Ewing's sarcoma-like primary melanoma	strong	10 months	living-with tumor
Primary-vertical growth phase	strong	lost to follow-up	unknown
Lymph node metastasis	positive	7 years	living-no tumor
Lymph node metastasis	strong	24 months	deceased
Dermal satellite metastasis	negative	4 years	living-with tumor
Lymph node metastasis	strong	4.5 months	deceased
Lymph node metastasis with phagocytosis	strong-negative around phagocytosis	6 years	living-with tumor
Satellite metastasis	positive	31 months	living-with tumor
Metastasis, with giant tumor cells	strong	17 months	deceased
Metastasis with giant cells	strong	26 months	deceased
Lymph node metastasis	postive	10 months	unknown

Wnt5a overexpression correlates strongly both to survival and time to the development of metastases. Time to the development of metastases is defined as the time elapsed from when the patient was first diagnosed with a primary melanoma to when the first metastasis was excised, as determined by pathology lab accession numbers.

cytology). When such areas could not be found, areas were examined in which the cells displayed features associated with less aggressive tumor behavior. These regions were then microscopically located on the Wnt5a antibody-stained slide, guided by the DAPI counterstain, and then imaged with a digital CCD camera for Wnt5a and DAPI staining. Wnt5a staining was graded in three levels, based on the intensity of the staining. Areas were called negative where no patterns of membrane-associated staining having an intensity of greater than 300 (arbitrary units) could be detected. Areas were graded as low to medium positive where typical membrane-associated staining could be detected with intensities of 500–1000. Areas with typical membrane-associated staining and intensities of 1000–4000 were graded as strongly positive.

The distribution of samples exhibiting areas of negative, weak, and strong Wnt5a staining in nevi, primary melanoma, and metastatic melanoma is shown in Figure 6A. It is easier to find cells with malignant features as tumor grade increases, and these cells frequently display strongly positive Wnt5a staining. In eight samples previously diagnosed as nevi, only two, both congenital nevi, showed Wnt5a staining. In both cases, the staining was confined to a small focus of cells showing multiple nucleoli, a hallmark of abnormal nevus cells. The ten tumors diagnosed as primary melanoma showed a wider gamut of staining, two were negative, two showed a few microfoci of strongly positive staining, two displayed moderately positive staining, and four had large zones of strongly positive staining. The eight samples of metastatic tumors showed an even greater fraction of cases with strong staining. One sample was negative, three showed regions of moderately positive staining, and five showed regions of strongly positive staining. These results are summarized graphically in Figure 6A. Examples of the cell morphology and Wnt5a staining patterns observed are displayed in Figures 6B–6J, and the details of this staining are described in the figure legend. The relationship of Wnt5a staining to tumor pathology and patient outcome is described in Table 1.

While these data strongly suggest that cell populations with strong Wnt5a expression become more pronounced in higher-grade tumors, the examples given do not represent a single natural history of a given melanoma, but only snapshots of many different histories. To provide a longitudinal view of disease in a single case, a series of samples from the course of the disease in a single patient who presented with acralentigious melanoma were examined. The primary tumor was located on thumb distal periungual skin. It was excised, but only to the limit of the superficial invasion, and was therefore reexcised to capture the leading margins of invasion. Over the next 2 years, metastatic melanoma to axillary lymph nodes was surgically removed. Cells from the region of superficial invasion stained mostly negative for Wnt5a, except within the superficial invasive component, where there was moderate positivity (Figure 7A). Cells from the next excision, at the leading edge of invasion, were strongly positive for Wnt5a (Figure 7B,arrow). The tumor samples from the early metastases to the axillary lymph nodes were mixtures of common morphological types of melanoma cells, epithelioid and sarcomatoid. The epithelioid cells showed lower levels of Wnt5a staining, while the sarcomatoid cells showed highly positive staining (Figure 7C). In keeping with the hypothesis that

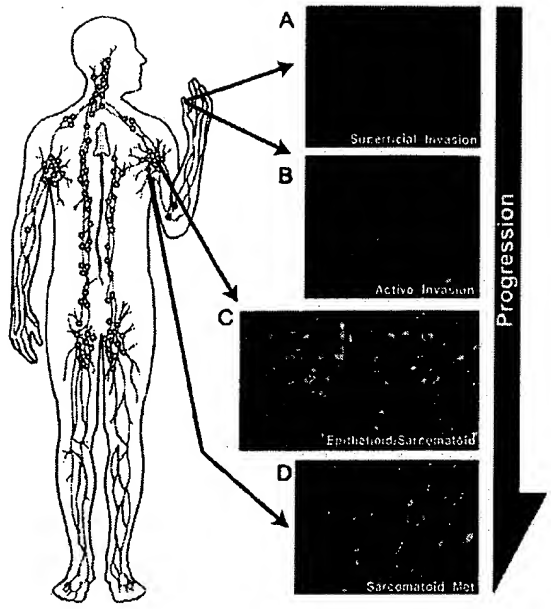

Figure 7. Natural history of an acralentigous melanoma

A–D: In this index case, not included in the above data, a patient presented with an acralentigious melanoma of the thumb. The tumor was excised and was composed mostly of keratin and some melanoma cells (**A**). The excision only captured the superficial edge of invasion, and the tumor cells at this edge were slightly positive for Wnt5a (**A**). Because the margins were not clear, the tumor was reexcised, and this time the leading edge of invasion was captured (**B**). The cells that were actively invading were strongly positive for Wnt5a (**B**). Six months later, this patient presented with a metastasis to the axillary lymph node. This tumor was composed of both epitheloid and sarcomatoid cells (**C**); however, the sarcomatoid cells were much more positive for Wnt5a than the epitheloid cells ([**C**], arrow). This became increasingly significant when the next metastasis, which occurred 1 month later, was entirely sarcomatoid in its composition (**D**).

stronger Wnt5a expression connotes higher invasive competence, the distant metastasis observed in this patient was completely sarcomatoid in its morphology (Figure 7D). While these initial in vivo staining results provide only a sketch of Wnt5a's localization in melanoma, the observations are consistent with what has been previously observed in molecular biological and biochemical studies of Wnt5a to date. Taken as a whole, our data support the notion that the Wnt5a/PKC pathway could play an important role in evoking the invasive phenotype of metastatic melanoma and that Wnt5a may be a potential marker of tumor progression.

Discussion

Previous studies of the gene expression profiles of melanoma indicated that *WNT5A* is an important marker that is highly correlated with enhanced motility and invasiveness (Bittner et al., 2000), suggesting that this gene might play a role in tumor metastasis. To further examine this possibility, we have examined the effects of this gene's expression in vitro and the distribution of its expression in melanoma in vivo.

Unlike other Wnt family members (e.g., Wnt1 and Wnt8), Wnt5a expression does not have a profound effect on β-catenin stabilization. Instead, Wnt5a activity has been shown to activate PKC (Kuhl et al., 2000). PKC has long been known to be associated with cytoskeletal changes and increases the motility of several types of cells, including melanocytes (Szalay et al., 2001). Moreover, PKC inhibitors have been shown to inhibit the ability of melanoma cells to invade through artificial basement membrane in vitro (Dennis et al., 1998). The many excellent studies implicating the PKC pathway as being central to invasion in melanoma elicit the question of how this pathway is induced. In this paper, we show that increasing or inhibiting Wnt5a expression produces a corresponding effect on PKC activation. In addition, our experiments provide direct evidence that increased Wnt5a expression is capable of increasing the in vitro motility and invasion of melanoma cells (Figure 4C). Frizzled-5 is implicated as the receptor activated by Wnt5a in this process by observations that disabling the receptor for Wnt5a leads to a reduction of both PKC activity and in vitro motility and invasion of melanoma cells (Figure 5). The detection of PKC isoform-specific phosphorylation patterns associated with the increased motility and invasion (Figure 4B) provides further definition of the likely route of this signal's transduction. Available data suggest that there may be a complex regulatory connection between Wnt5a and PKC. Prior published work (Jonsson et al., 1998) demonstrates that the increase or inhibition of PKC activity results in increased or inhibited Wnt5a expression, while the current observations show that the reverse is true as well. These observations suggest the possibility that the activities of Wnt5a and PKC drive a positive feedback loop, perhaps a Wnt5a autocrine loop, and that increases in the activity of either may result in increased melanoma motility.

In addition to displaying the expected characteristics of an inducer of metastatic behavior, Wnt5a is an attractive candidate on the basis of its normal function. In embryonic development, as neural crest cells migrate to the skin, they express high levels of Wnt5a, which results in increased morphogenetic movement in developing cells (Christiansen et al., 2000). When the cells reach their site of differentiation and become melanocytes, the expression of the Wnt5a gene drops to very low levels. Based on our current data and that of others (Bittner et al., 2000; Iozzo et al., 1995), it appears that reexpression of detectable Wnt5a is induced by a molecular event occurring during the progression of a melanocyte to malignancy, resulting in increased motile and invasive competence in those cells. The means of control of Wnt5a expression are not well elucidated, making it difficult to propose models of how increased Wnt5a expression might be achieved during progression. Factors known to alter Wnt5a expression include hepatocyte growth factor (Huguet et al., 1995), as well as c-Ha-ras, perhaps as a result of transduction of signals from the extracellular matrix (Bui et al., 1997). Further experimentation is required to support the notion that Wnt5a mediates interactions with the extracellular matrix in melanoma cells.

As with most signal transduction cascades, activation may be achieved by many paths. Another route to stimulation of PKC that affects cell motility is the Rho C pathway, which appears to be able to increase PKD (PKCμ) (Yuan et al., 2000), an isoform involved in cytoskeletal rearrangements and integrin activation (Palmantier et al., 2001). This stimulus has also been shown to increase the motility of melanoma cells (Clark et al., 2000). In addition, Wnt1 is able to regulate the expression of some Rho family members (Tao et al., 2001). The convergence of both the

Wnt5a and Rho C pathways to activate PKC provides a way for cellular motility to be conditioned by a variety of extracellular signals.

Early studies of Wnt5a RNA expression in tumors indicated that, on a gross level, many tumors showed increased Wnt5a expression relative to their normal tissue of origin, and that melanomas showed increased Wnt5a expression relative to skin (Iozzo et al., 1995). In this study, we show that the increased expression of Wnt5a in melanoma tumors is localized, occurring in cells at the site of active invasion and in cells showing morphological features associated with aggressive tumor behavior. This initial study sets the stage for further tumor progression studies of Wnt5a expression in melanoma, and its correlation to outcome will be examined via tumor microarrays.

In summary, our experiments demonstrate that Wnt5a, through its activation of PKC, contributes to the acquisition of a highly motile and invasive phenotype consistent with aggressive melanoma behavior. Wnt5a potentially mediates its effects by its ability to activate protein kinase C. It is possible that pharmacological inhibition of this pathway could be targeted to reduce the motility of these cells. Our findings are of particular interest, because unlike PKC, a molecule ubiquitous and active in all cell types, Wnt5a signaling does not appear to be common in adult organs. As such, Wnt5a signaling might be a potential therapeutic target for the inhibition of melanoma progression.

Experimental procedures

Cell lines

The human melanoma cell lines UACC 1273, UACC 647, M92-047, and UACC 930 were cultured in RPMI-1640 media supplemented with 10% fetal bovine serum (Hyclone), 100 U/ml penicillin G, and 100 U streptomycin. SW480 cells were a generous gift of Drs. Kenneth Kinzler and Bert Vogelstein (Johns Hopkins Medical Institution, Baltimore, MD) and were maintained in McCoy's 5A medium with 5% FBS. All cell cultures were incubated at 37°C in 5% CO_2/95% air, and the medium was replaced every second day.

Transfections

Cells were seeded in slide flasks and were allowed to reach 80% confluency. The cells were transfected with the Gateway destination plasmid PCDNA3-Wnt5a or the empty vector controls, using Lipofectamine Plus (GIBCO-BRL). After 6 hr of transfection, the medium was replaced with fresh serum-containing medium. For stable transfectants, medium was replaced after 48 hr with G418-containing medium, and transfectants were selected.

Cell adhesion assays

Cells were trypsinized, counted, and then seeded into 96-well plates at a density of 50,000 cells per well for 5, 15, and 30 min. Each time point was stopped by aspirating floating cells, rinsing the wells with PBS, and then fixing and staining the cells with 0.5% crystal violet in 50% methanol. Cell density was determined spectrophotometrically by dissolving the stain in the fixed cells with acetic acid and by measuring absorbance at OD 560 nM. Each time point was assayed in triplicate, and each experiment was repeated three times.

Immunoprecipitation and Western blotting

Phospho-Pan-PKC and Phospho PKC α/β II were obtained from Cell Signaling Technologies. Wnt5a antibody was obtained from R&D Biosystems. Cells were grown to 80% confluency and were then harvested on ice using cell lysis buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM ETA, 1% Triton X-100, 1× protease inhibitor cocktail [Boehringer Mannheim], and 1 mM sodium ortho-vanadate). Cells were dounce-homogenized and centrifuged at 12,000 rpm for 10 min. The supernatant was quantitated using the Pierce BCA protein quantitation assay. A total of 50 μg of each lysate was run out on SDS-PAGE 10% Tris-glycine Nu-PAGE gels and transferred onto 0.4 μm nitrocellulose. The membranes were probed with antibodies and were then visualized using the ECL system (Amersham).

Real-time PCR analysis

Real-time PCR analysis was performed using the Roche Light Cycler. Briefly, cells were grown to about 85% confluency and were then harvested for RNA using the Qiagen Rneasy Kit (Qiagen). RNA was analyzed for integrity using formaldehyde agarose gel electrophoresis and was quantitated. A total of 0.5 mg of RNA was then subjected to real-time RT-PCR using the SYBR Green 1 LightCycler RT PCR kit (Roche). The primers used for Wnt5a were: left primer; 5′-AGGGCTCCTACGAGAGTGCT-3′, and right primer; 5′-GACACCCCATGGCACTTG-3′. For Frizzled-5, the primers used were: left primer; 5′-CCTACCACAAGCAGGTGTCC-3′, and right primer; 5′-GGACAG GTTCTTCCTCGAAA-3′.

Immunohistochemistry

For β-catenin staining, cells were grown on glass slides and were allowed to reach 80% confluency. They were then fixed using methanol and were washed in PBS. Nuclei were permeabilized by incubating the cells in 0.4% NP40 in PBS (pH 7.4, calcium and magnesium free) for 10 min. Slides were washed and then blocked with horse serum for 1 hr, followed by an overnight incubation at 4°C in either β-catenin primary antibody (Transduction Laboratories) or in Wnt5a (0.2 mg /ml) or by phalloidin staining for actin (Molecular Probes). The cells were washed again with PBS for 30 min and were then probed with an FITC-conjugated secondary antibody for 1 hr (β-catenin). Cells were then washed, mounted in anti-fade, and examined under fluorescence.

For Wnt5a staining of paraffin-embedded tissue, paraffin-embedded tumors were sectioned and deparaffinized using a xylene, xylene, 100% ETOH, 95% ETOH, 75% ETOH, H_2O series, and antigens were retrieved by steaming samples in DAKO target retrieval buffer (DAKO) for 20 min. Sections were rinsed in PBS, were subjected to immunohistochemistry using a biotinylated Wnt5a antibody and a streptavidin-Cy5 secondary antibody, and were analyzed by immunofluorescence using a 12-bit CCD camera.

Invasion and motility assays

Scratch assays were performed by plating cells in slide chambers coated with fibronectin or collagen. After cells were allowed to attach and reach confluency, a scratch was made through the fibronectin or collagen. Photographs of cells invading the scratch were taken at the indicated time points. Invasion assays were performed using Matri-gel-coated Fluoroblok invasion chambers. The day before the assay, 8 mM fluoroblok filters (12 mM in diameter) were coated with 150 μl 80 mg/ml reconstituted basement membrane (Matrigel) and then placed in a sterile hood to dry overnight. A total of 16 hr prior to the assay, all cells to be assayed were serum starved. Prior to the assay, cells were treated with 5 mM Calcein-AM for 1 hr at 37°C. After this time, cell viability was examined using trypan blue exclusion, and then 50,000 cells were seeded onto the top of Fluoroblok filters. The total volume on the top of the filter was adjusted to 800 ml of serum-free medium. A total of 800 ml of the identical medium, with the addition of 10% fetal calf serum, was placed in the well beneath the filter to act as a chemoattractant. The cells were then placed in the 37°C-humidified incubator for 15 min, after which an initial reading was taken. All readings were performed on a Cytofluor 4000 with an excitation/emission of 480/530 and a gain of 50, using bottom read fluorescence only. Readings were taken every hour for 4–6 hr. All cell lines were assayed in triplicate in each experiment, and each experiment was repeated three times. ANOVA analysis was used to determine the statistical significance of the results, and standard deviations and p values are shown.

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